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Life Under Multiple Extremes: Exploring the Boundaries of Habitability on Earth

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Abstract

The habitable parameter space of a given environment is defined by the multiple constraints that restrict an organisms ability to propagate, and therefore by the maximum range of environmental conditions that life is able to tolerate. The habitability space within which biological processes occur is determined by the physicochemical conditions that restrict these processes. Natural habitats, however, often require organisms to tolerate multiple extreme conditions in combination, such as high or low temperatures, salinity, pH and pressure. Despite the existing laboratory and field data, and our understanding that natural environments can be best characterised by the net effect of multiple environmental parameters, basic studies on the interplay between concomitant environmental extremes on microorganisms is surprisingly limited. Subsequently the boundaries of the habitability space on Earth are yet to be confidently outlined and it is clear that these edges must be defined by the impacts of multiple environmental parameters.

Understanding how multiple environmental extremes effect microbial life is crucial in the assessment of the limits of life on Earth. In order to accurately determine the true limits of life it is necessary to examine the effect of multiple stress parameters on microbial growth. In this thesis, we examine the effects of salinity (NaCl), temperature and pH on propagation of a deep-sea hydrothermal vent microbe, *Halomonas hydrothermalis*, both in isolation and in combination to appropriately determine the impact of these stresses and the potential synergistic or antagonistic relationship between them. We chose these three factors because they are known to establish limits to life in natural environments and have been the focus of a substantial number of studies individually. Here we ask the question of whether a combination of these extreme environmental parameters approaches the physicochemical boundary of habitability space on Earth. These data show that multiple extremes, when combined, act to restrict the limits of life compared

to individual extremes. We see that changes in pH alters NaCl tolerance under optimal temperature conditions and under increased temperatures both acidic pH and temperature combine to further limit NaCl tolerance.

In addition, we explore the effect of multiple stresses of salinity, pH and temperature on microbial propagation using three facultative anaerobic strains (*Halomonas hydrothermalis*, *Escherichia coli* and *Carnobacterium pleistocenium*) with an aim to provide a better understanding of the energetic limits to life by assessing the maximal growth values attained under different modes of respiration. It is known that energy yields differ between aerobic and anaerobic respiration and the presence of environmental factors such as pH, NaCl and temperature can significantly alter their efficacy, yet few laboratory studies have been done to systematically explore the interactions of three or more stresses on the limits of microbial growth under both aerobic and anaerobic conditions. Here we demonstrate these facultative anaerobic microbes display significantly different tolerances to a combination of stresses of pH, NaCl concentration and temperature when cultivated under aerobic and anaerobic conditions. These data show increased tolerance to higher saline concentrations under anaerobic culture conditions when compared with aerobic growth for two of the strains tested. Additionally, we demonstrate complex interactions between acidic pH and NaCl concentration under increased temperatures where we see anaerobic cultures yield higher growth values than aerobic cultures under a combination of these stresses. These data have significant implications when considering the effect a rise of atmospheric oxygen during The Great Oxidation Event may have had on Earths anaerobic microbial population. Here we demonstrate an essential need to assess the habitability of natural environments with a deeper understanding of the interplay between concomitant physicochemical parameters with a particular focus on cellular energetics.

Furthermore, pressure is a fundamental parameter of Earths biosphere that has played a key role in the evolution and distribution of life on Earth, with higher than atmospheric pressure environments predicted to be major habitats for prokaryotic life that exceed numbers found in other components of the biosphere. The effect of high pressure imposed on organisms in addition to other environmental factors such as salinity and pH are yet to be fully understood. The necessary adaptations to deal with these extremes individually are well established, yet the potential synergistic or antagonistic nature of a combination of these stresses in high pressure environments requires

a more robust understanding of the physical constraints imposed on life under high pressures conditions. Here we demonstrate that when cultivated under simulated hydrostatic pressures of 50-bar and 150-bar, the model organism *H. hydrothermalis* displays higher growth than when cultured under atmospheric pressure conditions over a range of salinities and pH values.

These data show multiple extremes potentially restrict the boundaries of the biosphere more than single stresses imposed alone. Thus, the boundary space for life in natural environments may be smaller than research into the limits of life in individual extremes would suggest and habitable environments may in fact be less pervasive throughout the universe than previously thought. To determine the window of tolerance to environmental pressures imposed on microbial extremophiles is an essential tool in furthering our understanding of the evolution and diversification of life on Earth, and in making clearer the potential habitability of other planetary bodies, both within and beyond our Solar System.

Lay Summary

Habitability is a definition applied to environments to determine its ability to host life. We can think about the habitability of life on Earth as existing within a boundary that is controlled by the environmental conditions that life experiences. These include environmental conditions such as high and low temperatures, NaCl (%), pressure and pH. Advances have been made to further our understanding of how life deals with extremes of these conditions, and these investigations are frequently used to determine the habitability of an environment based on an organisms capacity to cope with a single extreme experienced in isolation. Natural environments, however, are better characterised by the effect of multiple environmental conditions, yet our understanding of how multiple extremes effect habitability is surprisingly limited.

This thesis studies the response of bacteria to combinations of conditions to help further our understanding of the limits of life under multiple extremes. Research of this nature will not only inform us of how the boundaries of habitability of Earth are shaped by combinations of extreme conditions but will also make clearer the potential habitability of environments on other planetary bodies, both within and beyond our Solar System.

The first section of this thesis investigates the effects of salt (NaCl), temperature and pH on the growth of a deep-sea hydrothermal vent bacterium both in isolation and in combination, to ask the question of whether a combination of these common environmental conditions approaches the boundary of habitability on Earth. This work demonstrates that extremes of NaCl concentration, pH and temperature act to define narrower limits to microbial growth than individual extremes. These findings show that to assess the habitability of an environment based on an organisms ability to cope with one extreme condition is to do so without taking into account the combined effect of multiple extremes and highlights a need for a more robust definition of the boundaries of habitability.

To further our understanding of the habitability of extreme environments it is necessary to consider the energetic cost of dealing with multiple extreme conditions. For an organism to grow in a given environment it must be able to meet the energy requirements necessary to cope with the environmental stresses imposed it. Aerobic organisms survive and grow in the presence of oxygen, using it for respiration, and anaerobic organisms do not require oxygen for growth and employ compounds other than oxygen in their respiration. These modes of respiration are known to produce different amounts of energy for the organism to use in dealing with extreme conditions. Facultative anaerobic organisms are capable of growing in the presence or absence of oxygen and are ideal candidates for examining how the limits of life differ for these different modes of respiration. The second section of this thesis demonstrates that the availability of oxygen may release some organisms from the low energy constraints of anaerobic respiration, however, under certain conditions there was little or no improvement in growth. This has implications when considering the rise of oxygen during The Great Oxidation Event and suggest this newly exploitable energy source may have extended the limits to life under certain environmental conditions but restricted it in others.

To gain a better understanding of how a physical environmental parameter affect microbial growth under multiple extremes it is necessary to consider the effect of high pressure and the interaction with other conditions. Pressure is a fundamental property on Earth, and the majority of life occupies environments that are subject to higher than atmospheric pressure conditions. In response to a lack of data on the effect of pressure on the limits of life in multiple extremes, the final section of this thesis explores the growth limits of a deep-sea hydrothermal vent isolate under a combination of NaCl (%) and pH stress both at atmospheric pressure and under simulated hydrostatic pressures. The findings show a significant synergistic relationship between NaCl (%), pH and high pressure that extends the boundary of habitability for our model strain. This has implications for understanding the potential for habitable environments on other planetary bodies such as Europa and Enceladus, which are suggested to experience Earth-like deep-sea pressures and moderate temperatures.

Collectively, these findings show that to determine the boundaries of habitability based solely on an organisms ability to propagate within the boundaries of one extreme parameter is to do so without taking into account the combined effect of concomitant stresses within natural habitats. To provide a better definition

of the true limits to life requires the boundary space for life to be mapped with consideration of the interactions between multiple extremes present in the environment.

Declaration

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification except as specified.

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Contents

List of Figures

List of Tables

Chapter 1

Introduction

The habitability of an environment is defined by the multiple constraints that limit organismal propagation, and therefore by the maximal range of environmental parameters tolerated by at least one organism (Cockell et al., 2016; Harrison et al., 2013). The impact of individual extremes and their effects on microbial life have been extensively studied and are commonly referenced when determining the habitability of a particular environment. Natural habitats, however, often require organisms to deal with multiple extreme conditions in combination, such as desiccation, high or low temperatures, high salinity and ultra-violet radiation (Dartnell, 2011; Harrison et al., 2015b; Merino et al., 2019; Pikuta et al., 2007; Rothschild and Mancinelli, 2001).Indeed, environments on Earth are best characterised by a wide range of parameters that may prove synergistic or antagonistic due to shared adaptive mechanisms, for example high pressure and temperature in the deep-sea, salinity and temperature in sea-ice brine veins, or NaCl concentration and pH in saline lake systems. Despite this, there have only been only a hand full of investigations into the mechanisms that facilitate life in conditions of simultaneously occurring extremes. The lack of information regarding the growth and physiological response of microbes to concomitant extremes exposes a need for a more detailed understanding of the growth response of organisms under combined stresses. This work aims to address this gap and test the hypothesis that the combination of these extreme environmental parameters restricts the growth limits of the organism more than the individual extremes.

For an organism to propagate in a given environment it must be able to

meet the energy requirements necessary to cope with the environmental stresses imposed on it to support a functioning biochemistry (Cockell and Nixon, 2013). When life has access to liquid water the availability of metabolic energy may be the most fundamental property governing environmental habitability and ecosystem structure (Hoehler, 2007; McCollom and Amend, 2005). In theory, if an organism's energy resources are allocated to the adaptive mechanisms used to deal with one stress parameter, there will be fewer resources available to cope with other environmental stressors imposed (Bowers et al., 2009). One of the most profound changes in the history of life on Earth is the transition of its atmosphere from anaerobic to aerobic (Kopp et al., 2005; Sessions et al., 2009), yet our understanding of how this introduction of newly exploitable chemical energy may have altered the limits of microbial life is limited. Due to this, we aim to quantify the combined effect of stresses of salinity, pH and temperature on three facultative anaerobic organisms under aerobic and anaerobic conditions with the objective to better understand the role of oxygen in potentially releasing life from the constraints of low-energy anaerobic conditions.

Further to this, high pressure environments are ubiquitous on Earth with the deep-sea and subsurface habitats predicted to harbour numbers of prokaryotes that far exceed numbers found in other components of the biosphere (Cario et al., 2019; Fang and Bazylinski, 2008; Whitman et al., 1998). Higher than atmospheric pressure conditions are a fundamental parameter of Earth's biosphere due to high pressure environments being host to prokaryotic life that exceeds numbers found in other components of the biospehere, and has played a key role in the evolution and dissemination of life on Earth (Jannasch and Taylor., 1984; Nisbet and Sleep, 2001; Whitman et al., 1998). The effect of high pressure imposed on organisms in addition to other environmental factors such as salinity and pH are yet to be fully understood. The necessary adaptations to deal with extremes of salinity and pH individually are well established (Baker-Austin and Dopson, 2007; Konings et al., 2002; Krulwich et al., 2011; Oren, 2006), yet the potential synergistic or antagonistic nature of a combination of these stresses in high pressure environments requires a more robust understanding of the physical constraints imposed on life under high pressures conditions. Understanding the interplay between multiple stresses under physical constraints provides further insight into characterising the net effect of multifarious environmental extremes on microbial viability.

To assess the boundaries of physical and chemical limits of life on Earth helps

provide answers to far-reaching scientific questions about how life first developed on Earth, the conditions that allowed for life to evolve and thrive, and gives insight into the potential habitability of extraterrestrial environments. In addition to implications in astrobiology, understanding how these physicochemical boundaries of life shape the habitability space on Earth is important in many fields of research including biotechnology (Nichols et al., 2000; Rothschild and Mancinelli, 2001; Tanaka et al., 2001), environmental science (Harrison et al., 2013), climate change (Waldron et al., 2007) and in potential medical applications such as antibiotic resistance (Harrison et al., 2017).

1.1 Thesis Scope and Objectives

The gap of information regarding the growth and physiological response of microorganisms to multiple extreme conditions exposes a need for further research into microbial growth under concomitant extremes. The primary objective of this thesis was to further our understanding of the effect of multiple environmental extremes on microbial propagation. The main research questions addressed and hypotheses for each data chapter were as follows:

Question 1: How does a combination of extreme environmental parameters influence the physico-chemical limits of life compared to individual extremes?
The effect of individual environmental stresses of NaCl, pH and temperature are well characterised and it is clear there is considerable overlap in the mechanisms employed to deal with the stresses individually. We hypothesise that the combination of these extreme environmental parameters further restricts the growth limits of the model organism more than each stress experienced individually.

Question 2: Does the presence of oxygen release life from the constraints of low energy anaerobic conditions and change the limits of life under multiple extremes?
Energy transduction in the cytoplasmic membrane is driven by environmental factors such as pH and NaCl, and it is established that anaerobic respiration produces lower yields of ATP than aerobic respiration. We hypothesise that aerobic respiration allows for a broader range of cell division under extreme conditions of NaCl, pH and temperature than aerobic respiration due to higher

energy yields allowing for increaces capacity to cope with environmental stresses.

Question 3: Do physical environmental parameters, particularly pressure, govern an organisms tolerance to multiple extremes?
Previous studies have demonstrated the synergistic effect of high pressure and other environmental factors, such as temperature, on microbial survival. We hypothesise that increased simulated hydrostatic pressure will extend the model organisms capacity to deal with variations in NaCl concentration and environmental pH.

1.2 Thesis Outline

Chapter 2 provides a detailed assessment of our current understanding of life under extreme conditions, highlighting the known boundaries of habitability on Earth with respect to individual extreme conditions and discussing the current understanding of life under individual extremes. It also examines microbial adaptations to deal with environmental stresses both individually and in combination, providing insight into whether these limits should be considered in combination rather than individually, and whether they may be defined by thermodynamic or physical parameters. Chapter 3 provides the general materials and methods employed throughout this research. A more in-depth explanation of materials and methods used for each chapter are discussed in each results chapter respectively.

Chapter 4 presents research into the effect of multiple extremes (supra-optimal temperature, pH and salinity) on the propagation of a model organism *Halomonas hydrothermalis* and addresses Question 1 of the thesis objective. Chapter 5 addresses question 2 of the thesis objectives and reports research concerning the bio-energetic cost of life under multiple extremes (supra-optimal temperature, acidic pH and salinity) by exploring the variation between aerobic and anaerobic respiration on an organism's capacity to cope with environmental extremes using three facultative anaerobic bacterial strains (*H. hydrothermalis*, *Escherichia coli* and *Carnobacterium pleistocenium*). Chapter 6 details the effect of simulated hydrostatic pressure on the propagation of *H. hydrothermalis* under a combination of variations in salinity and pH and addresses question 3 of the thesis objectives.

Chapter-specific limitations and suggestions for future directions are provided at

the end of each data chapter.

The principal findings of these chapters are summarised in Chapter 7, which details the general conclusions drawn from these studies and reports potential future directions.

Chapter 2

Background

2.1 Introduction

The aim of this thesis is to explore the boundaries of habitability under combinations of environmental extremes. Throughout this thesis a number of model prokaryotic strains are used that cover a wide range of tolerances to the environmental stresses examined, and photosynthetic prokaryotic strains will not be used at any stage, therefore light availability will not be a limiting factor. In the first instance, the focal point will be to examine the effects of multiple stresses of salinity, pH and supra-optimal temperatures both individually and in combination on microbial propagation. Though considerable research has been done to assess the effect of these stresses individually, the combined effects of their influence on microbial propagation is yet to be fully understood (Harrison et al., 2013). Following this, we examine the effect of these stresses on maximal growth values for three facultative anaerobic microbes with an aim to advance our understanding of the additional impact of energy expenditure when coping with concomitant extremes. Aerobic and anaerobic respiration are known to differ in their energy yields (King, 2005; Unden and Bongaerts, 1997) and aerobically respiring organisms have been shown to display a broader tolerance range to environmental extremes (Harrison et al., 2015b), yet few laboratory studies have been done to systematically explore the interactions of three or more stresses on the limits of microbial growth under both aerobic and anaerobic conditions. Finally, we aim to provide a further understanding of the effect of these multiple extremes under the influence of the proposed physical environmental stress of

simulated hydrostatic pressure. Pressure is a fundamental parameter on Earth and higher than atmospheric pressure environments are predicted to be a major habitat for microorganisms that far exceed the extent of other environments on Earth (Cario et al., 2019; Fang and Bazylinski, 2008; Whitman et al., 1998), yet little is understood about the potential synergistic or antagonistic nature of a combination of these stresses in high pressure environments.

This chapter discusses the background literature relevant to the focus of this thesis. Here I discuss the boundaries of habitability on Earth and outline the currently established limits under extremes of salinity, pH, temperature, and pressure imposed individually, outlining the adaptive mechanisms for dealing with these extremes. Additionally, I discuss the previously established impact of multiple extremes on microbial life giving an overview of how the adaptive mechanisms for extreme conditions may prove synergistic or antagonistic to other extremes imposed in the natural environment. Furthermore, I explore the history of oxygen on early Earth and discuss the role that the rise of atmospheric oxygen may have played in releasing microbial life from the constraints of anaerobic modes of energy acquisition and subsequently changing life's limits to multiple extreme conditions.

2.2 Boundaries of the Biospace

We can think about the habitability of life on Earth as being bound by a parameter space within which all life persists. The edges of this biospace are controlled by the physicochemical conditions that determine the capacity for life to persist within this boundary space (Dartnell, 2011; Merino et al., 2019; Pikuta et al., 2007; Rothschild and Mancinelli, 2001). This concept is subject to interpretation, as the boundaries can be characterised depending on the limits under investigation (e.g. survival, propagation, metabolic activity), however, to call into question the definition of habitability is not the aim of this thesis. Rather, for the purpose of this study, habitability is defined as an environment capable of supporting at least one organisms ability to survive, grow and reproduce (Cockell et al., 2016).

When considering this biospace, much of the life we more commonly recognise occupies the central area where conditions are anthrocentrically determined. That is to say, this area of the biospace is favourable to us as humans and the conditions

we find ideal, and indeed most multicellular life and much of the microbial world is able to grow and reproduce (Figure 2.1). As we move outwards and begin to approach the edges of the biospace, conditions become increasingly extreme and we find that microbes dominate and diversity is greatly reduced to those with the specialist biochemical adaptations to deal with the stress of extreme conditions. Microbes are single cell organisms that can be prokaryotic (the bacteria and archaea) or eukaryotic (for example algae and yeast), and prokaryotic life has dominated most of the evolutionary history of life on Earth (Cockell, 2020; Marques et al., 2008). Extremes that commonly restrict the biological processes necessary for life to persist include temperature, salinity, pH, pressure, UV radiation and nutrient availability (Dartnell, 2011; Harrison et al., 2015b; Merino et al., 2019; Pikuta et al., 2007; Rothschild and Mancinelli, 2001)

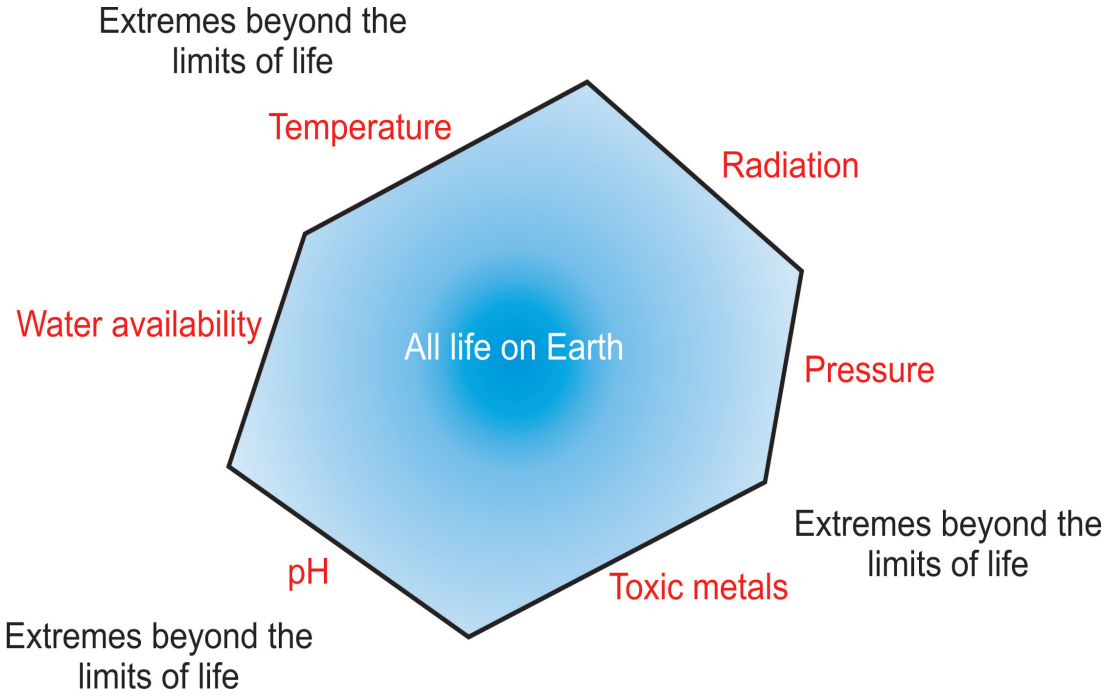

Figure (2.1) *A two-dimensional example of Earth's biospace defined by an example set of chemical and physical extremes. The central area of this space is occupied by the vast majority of life and approaching the boundaries we find life becomes less diverse and is dominated by microbes. (Cockell, 2020).*

Extensive research examining the effect of these extremes individually has been conducted over recent decades on a large number of organisms (Aston and Peyton, 2007; DasSarma and DasSarma, 2001; Jaenicke and Sterner, 2006; Konings et al., 2002; Krulwich et al., 2011; Méndez-García et al., 2015; Merino et al., 2019; Mesbah and Wiegel, 2012; Oren, 2006; Tolner et al., 1997). The resulting information of these studies are often applied to extreme environments to

determine their habitability. Our current understanding of the limits of life under extreme conditions is based on an organisms capacity to cope with particular physicochemical extremes in isolation and rarely the simultaneously occurring stresses that more commonly define an extreme environment. This thesis aims to address this lack of information regarding the growth and physiological response of microbes to concomitant extremes with the aim of addressing the potential for synergistic or antagonist relationships between extremes. To provide a better understanding of the limits of life under multiple extremes would allow us to gain new insight into the complex interplay of concomitant extremes at the boundaries of life.

2.3 The Limits of Life

To better understand the potential synergistic or antagonistic relationship between concomitant extremes, here I briefly summarise our current knowledge of known habitats for each stress parameter relevant to this thesis and explore the mechanisms employed to deal with these extremes. Extremes of NaCl ($), pH and temperature are relevant to Chapters 4 and 5. Extremes of NaCl (%), pH and pressure are relevant to Chapter 6. Detailed reviews have been composed with respect to each of the following extremes [see Oren (2006) for salinity, Krulwich et al. (2011) for pH, Jaenicke and Sterner (2006) for high temperatures, Siddiqui et al. (2013) for low temperatures and Oger and Jebbar (2010) for pressure]. For the purpose of this thesis, these adaptive mechanisms will not be discussed in detail, rather the major adaptive mechanisms will be explored with an aim to address the potential overlap between environmental extremes imposed and the respective mechanism for dealing with these extremes in combination.

2.3.1 Salinity

The worlds oceans cover around 70% of the Earths surface and contain approximately 35 g/L dissolved salts (3.5% NaCl wt/vol] with vastly higher concentrations, up to and exceeding saturation, found across the globe that provide potential habitats for extreme microorganisms (Oren, 2006; Ventosa et al., 1998). Environments exceeding the dissolved NaCl concentration of seawater are defined as hypersaline (Vellieux et al., 2014). Examples of hypersaline

environments include the Great Salt Lake, The Dead Sea, artificial salterns used in salt mining from seawater and salt inclusions (Benison et al., 2007; DasSarma and DasSarma, 2001; Lee et al., 2018; Ollivier et al., 1994; Scambelluri et al., 1997; Ventosa et al., 1998). NaCl concentrations in these environments vary but at times are saturated or oversaturated (Lee et al., 2018). It is clear that salinity is a ubiquitous characteristic of the Earth's biosphere. It is known to be one of the primary shapers of microbial communities (Lozupone and Knight, 2007) and though it is known that all life forms require salts, organisms capable of tolerating these high levels of NaCl are termed halophiles. Halophiles are classified in accordance to their respective level of NaCl requirement, from those capable of growth in slightly increased NaCl concentration to those capable of withstanding extreme levels of NaCl in the environment. The specific definitions for what constitute a halophilic organism differs between authors and the boundaries for specific definitions of the classifications of halophiles varies, though there is considerable overlap, see Oren (2006) and DasSarma and DasSarma (2001). The parameters outlined in Oren (2006) are generally considered the accepted terms for the classifications of halophiles (Table 2.1). At the time of this study, the current record holding halophilic organism is *Halarsenatibacter silvermanii* strain SLAS-1T, which has an optimal growth at saturation (35% NaCl [wt/vol]) and requires a minimum of 20% NaCl for growth (Blum et al., 2009). Interestingly, *H. silvermanii* has an optimal growth temperature of 44°C (range 28 to 55°C) and an optimal pH of 9.4 (range pH 8.7 to9.8) (Blum et al., 2009), demonstrating a need to further understand the potentially synergistic relationship between overlapping mechanisms when dealing with these environmental extremes in combination.

Table (2.1) *Classification of Halophiles (Oren, 2006).*

Category	Properties	Examples
Non-halophilic	Grows best in media containing less than 0.2M NaCl	Most freshwater bacteria
Slight halophilic	Grows best in media containing 0.2 to 0.5M NaCl	Most marine bacteria
Moderate halophile	Grows best in media containing 0.5 to 2.5M NaCl	*Salinivibrio costicola*
Borderline extreme halophile	Grows best in media containing 1.5 to 4.0M NaCl	*Halorhodospira halophila*
Extreme halophile	Grows best in media containing 2.5 to 5.2M NaCl	*Halobacterium salinarum*
Halotolerant	Non-halophile which can tolerate NaCl; if the growth range extends above 2.5 NaCl, it may be considered extremely halotolerant	*Staphylococcus aureus*

Previous studies have shown that halophilic species have developed unique ways of dealing with high levels of NaCl in their environment (DasSarma and DasSarma, 2001; Oren, 2006, 2008). When challenged with high NaCl concentrations, organisms are required to maintain osmotic balance between their cytoplasm and

the environment, and there are two strategies that are considered fundamental within the microbial world. One mechanism is the salt-in strategy, which involves maintaining high concentrations of potassium ions within the cell and requires extensive adaptations of the intracellular systems, which can only be achieved in a long and complex evolutionary process (Oren, 2006, 2008). As osmolality in hypersaline environments can be detrimental to cells, this method prevents loss of water to the external medium through osmotic balance (DasSarma and DasSarma, 2001). Despite its relatively low energy costs compared with the alternative method, only a small number of halophiles employ this strategy as a means of osmotic balance (Oren, 2008). The second mechanism is the low salt-in or compatible-solute strategy, which involves maintaining concentrations of NaCl within the cytoplasm lower than that of the outside medium with the use of organic compatible solutes (Oren, 2011; Roberts, 2005). These compatible solutes are thus termed due to their physicochemical properties making them physiologically compatible with cellular machinery (Bremer and Krämer, 2019) and thereby maintain osmotic equilibrium without the need for specially adapted intracellular systems (Oren, 1999). This method is energetically costly, as organisms that use organic solutes to balance the osmotic pressure of the cytoplasm must also expend energy in ion pumping to keep intracellular ionic concentrations low, and to counteract the diffusion of inorganic salts through the membrane (Roberts, 2005). Despite the higher energetic cost of the low salt-in strategy, this method is far more wide-spread in nature (Oren, 2008). These essential adaptations to saline conditions requires the regulation of phospholipid composition to adjust membrane permeability and remain stable under a wide range of sodium ion concentrations, and this strategy is linked directly with proton permeability (Konings et al., 2002; Padan, 2001) and is ultimately a function of growth temperature (Oren, 2008; Van De Vossenberg et al., 1999).

2.3.2 pH

Environments with extremes of pH are found across the globe. To deal with extremes in pH, all organisms must maintain near neutral intracellular pH for fundamental cellular biochemistry to function (Krulwich et al., 2011). Maintaining a neutral intracellular pH requires the movement of ions across the cytoplasmic membrane which is dealt with in two distinct ways; pumping of ions outside of the cell in acidic environments and movement of ions to within the cell in alkaline environments (Konings et al., 2002).

Acidic environments are common in nature such as such as The Rio Tinto River in Spain, a naturally acidic drainage environment where iron sulfide reacts in water resulting in sulfuric acid creating am acidic river which has an average pH of 2.3 (Barrie Johnson and Hallberg, 2008; Cockell, 2020; Sánchez-Andrea et al., 2011), hydrothermal vent fluids known to be moderately acidic which also come into contact with sulfur from volcanic activity (Cockell, 2020; Ding et al., 2005; Tan et al., 2017), mine drainage systems and subsurface ecosystems are known to exhibit significantly acidic conditions, and pH is known to be a major determining factor in microbial community composition (Czop et al., 2011; Jin and Kirk, 2018; Miettinen et al., 2015; Nordstrom et al., 2000; Pankova and Konyushkova, 2013; Roadcap et al., 2006; Waldron et al., 2007). Many of these environments are also subject to extremes in pressure, salinity (Benison et al., 2007; Waldron et al., 2007; Zaikova et al., 2018) and temperature (Pankova and Konyushkova, 2013), further raising questions about the multiplicative effect of these extremes on life in these habitats.

Under acidic conditions organisms must maintain a neutral intracellular pH by actively pumping protons from the cytoplasm across the membrane, and the membranes of acid tolerant organisms are highly impermeable to restrict proton influx into the cell (Baker-Austin and Dopson, 2007). Consequently, a careful balance of proton influx and the rate of proton pumping outwards is necessary to maintain a suitable proton motive force (PMF), which is an essential electrochemical gradient of protons across the membrane (Baker-Austin and Dopson, 2007; Krulwich et al., 2011). The current record holders for life at acidic pH are two species of archaea (*Picrophilus oshimae* and *Picrophilus torridus*) isolated from hot springs in Japan (Schleper et al., 1996). These organisms grew only below pH 3.5 with an optimal growth of pH 0.7 and significant growth was observed as low as pH 0, and under a temperature of 60°C (Schleper et al., 1996). These strains demonstrate a potential synergistic relationship between overlapping mechanisms to environmental extremes of acidic pH and high temperatures, raising questions about our understanding of the boundaries of habitability in these environments.

Alkaline environments exist around the world in areas where water interacts with specific volcanic rocks (Marques et al., 2008), such as Mono Lake in California where salts accumulate within a shallow water body resulting and a pH of 10 (Cockell, 2020; Humayoun et al., 2003) and lakes Magadi and Bogoria in Kenya (Cioni et al., 1992; Jones et al., 1977; Zhilina et al., 1998). High pH

tolerant organisms can grow at pH >8 (Pikuta et al., 2007), and much less is understood about organisms that tolerate these high pH values than those in low pH environments. Similarly to low pH, organisms at high pH must also maintain a neutral intracellular pH, and do so through the use of membrane proteins called Na^+/H^+ antiporters, which are crucial in both pH and Na^+ homeostasis (Konings et al., 2002; Padan, 2001). The current known record holder for life under alkaline conditions are *betaproteobacterial* strains isolated from pH 11.6 serpentinizing springs in California and are thus termed *Serpentinomonas* (Suzuki et al., 2014). Strain B1 of the three strains isolated was able to grow at pH 12.5, with an optimal of pH 11, and has an optimal salt (NaCl) of 0% with an extremely narrow range of 0 to 0.05% NaCl (Suzuki et al., 2014). Considering many highly alkaline environments are also characterised by high salt concentrations (Cioni et al., 1992; Czop et al., 2011; Zhilina et al., 1998), and many high pH tolerant organisms occupy environments that are also rich in sodium ions (Konings et al., 2002), the capacity for this strain to grow at such high pH and be susceptible to low saline conditions further demonstrates the importance of understanding the multiplicative effect of extremes on microbial propagation and distribution.

2.3.3 Temperature

Temperatures on Earth are hugely varied. The lowest ever recorded air temperature is -89.2°C at Vostok Station in Antarctica (Turner et al., 2009), with lower temperatures proposed at the snow surface, located on the Antarctic Plateau above Vostok, to reach -90°C and below (Scambos et al., 2018). Much of the Earth can be classified as a cold biosphere, which may in fact represent one of the largest proportions of the worlds biosphere. As previously mentioned (2.3.1.), much of the Earths surface (∼70%) is covered with ocean, and the temperature of much of the ocean is approximately -1 to 5°C, and polar regions including lands within the Arctic circle and Antarctica represent roughly 20% of the Earth's terrestrial surface area (Cowan and Stafford, 2007). A significant portion of these areas are permafrost environments with approximately 55% of Russia and Canada, 85% of Alaska and most likely all of Antarctica potentially playing host to permafrost microbial communities (Rivkina et al., 2000). The current known record holder for organisms at low temperature extremes are a natural population of bacteria isolated from permafrost in Siberia that was metabolically active between 5 and -20°C (Rivkina et al., 2000). It is important to note, however, that metabolic activity is not unquestionably linked with microbial propagation and

may only be at levels sufficient for survival (Morita, 1997). Our full understanding of the lower limits to microbial growth are yet to be fully understood.

Microbial life has adapted a variety of ways to deal with low ambient temperatures. One of the primary adaptations to changes in environmental temperatures is through altering the composition of the cytoplasmic membrane, which is necessary to maintain a liquid crystalline state (Clarke et al., 2013; Russell and Fukunaga, 1990). A decrease in ambient temperature can have physical effects on the properties of microbial membranes that diminishes membrane fluidity through reducing the packing efficiency of fatty acids that make up the membrane (Chattopadhyay, 2006; D'Amico et al., 2006; Konings et al., 2002; Siddiqui et al., 2013). This decrease in fluidity, and subsequently permeability, of the membrane can prove detrimental to cellular processes, and psychrophilic organisms must adjust their membrane fatty acid profile to maintain optimal fluidity. This is achieved by increasing the proportion of unsaturated fatty acids, which introduces kinks into to the phospholipids and results in more fluid membrane structure (Konings et al., 2002; Russell, 2008).

High temperature environments on Earth range from desserts reaching up to 80°C and geothermal hot springs that range in temperatures as high as 50 and 100°C (Cioni et al., 1992; Clarke, 2014) to deep-sea hydrothermal vent systems. Deep-sea hydrothermal vent systems produce vast amount of heat under the combined influence of hydrostatic pressure, which allows for liquid water to reach temperatures up to, and exceeding, 400°C (Koschinsky et al., 2008; McDermott et al., 2018). High temperature tolerant organisms have primarily been isolated from deep-sea hydrothermal vents where water is able remain liquid at temperatures exceeding 100°C due to high hydrostatic pressures (Clarke, 2014; Cockell and Nixon, 2013; McDermott et al., 2018). One of the main adaptations in these microbes is of the cytoplasmic membrane. Under high temperatures the increased motion of the lipid molecules that comprise the membrane leads to them becoming overly fluid and proton permeability is dramatically increased (Jaenicke and Sterner, 2006; Konings et al., 2002). The membranes of these organisms are known to be contain an abundance of saturated fatty acids which increase rigidity (Jaenicke and Sterner, 2006). By increasing rigidity and reducing membrane permeability, these organisms are better able to maintain crucial proton and sodium ion gradients across the membrane, thus preserving essential cellular function. At the time of this study, the current record holding organism for growth at high temperatures is *Methanopyrus kandleri* strain 116 which is capable

of growth at 122°C (Takai et al., 2008). This remarkable organism is able to grow at these high temperatures due to the influence of high hydrostatic pressure, under which, the temperature limit for cellular proliferation was extended from 116 to 122°C (Takai et al., 2008). This further demonstrates the need for an increased understanding of the combined influence of environmental extremes and more robust definition of the boundaries of life under combined extremes.

2.3.4 Pressure

Pressures experienced by microbial life on Earth ranges from $\sim$ 1 bar at sea level to the deep ocean pressures exceeding 1000 bar (Mota et al., 2013; Picard and Daniel, 2013). As a parameter for life, pressure is widely considered the most ubiquitous, with the deep-sea (considered within the range of >1000 m depth and >100 bar pressure (Bartlett, 1999; Mentre and Hui Bon Hoa, 2001) accounting for approximately 65 to 75% of the total ocean, and believed to host approximately 62 to 88% of Earths biosphere (Cario et al., 2019; Fang and Bazylinski, 2008). Additionally, the deep subsurface, such as marine sediments and terrestrial habitats below 8 m, are major environments for life that exceed numbers found in other components of the biosphere (Whitman et al., 1998). Organisms that are capable of withstanding these higher than atmospheric pressures are termed piezophiles (or sometimes barophiles) (Canganella and Wiegel, 2011).

One of the best, and most fundamental, examples of how organisms deal with changes in pressure is through changes in the fluidity of microbial membranes. As with low temperatures, high pressure increases the packing of the fatty acids that make up the membrane (Mota et al., 2013; Oger and Jebbar, 2010). To cope with this increased packing, piezophilic organisms increase the number of unsaturated fatty acids in their membrane to increase fluidity and maintain a liquid crystalline state (Bartlett, 2002; Oger and Jebbar, 2010) (Figure 2.2).

This increase in unsaturated fatty acids can occupy a proportion as high as 70% of the total membrane profile (Fang et al., 2010). As has already been explored in section (2.3.3.), pressure has a particular influence on microbial growth at high temperatures. Indeed, the current known record holder for growth under higher pressure is *Thermococcus piezophilus* which displays optimal growth at 50 MPa (500 bar) and at a temperature of 75°C (Dalmasso et al., 2016). Additionally, with much of the deep-sea subject to low temperatures, it is not surprising that the adaptive mechanisms for dealing with both high pressures and low temperatures

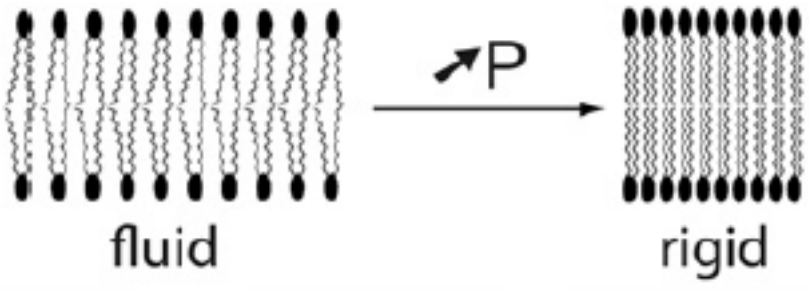

Figure (2.2) *Graphical representation of the effect of pressure on lipids in the membrane.*
Lipids become less fluid under increased pressure (P) and the membrane becomes less permeable to water and ions (Oger and Jebbar, 2010).

have considerable overlap (Bartlett, 1999; Fang et al., 2010; Winter and Jeworrek, 2009). Some environments exhibit pressures lower than atmospheric levels, such as high-altitude mountains and low Earth orbit, though these are not thought to affect microbial viability (Merino et al., 2019), however data is scarce and the effect of low-pressure environments on microbial survivability is not understood as the definition of low-pressure ecological niches on Earth that harbour low-pressure adapted organisms is difficult. Further research into the effect of microbial survivability under low pressures, in addition to multiple environmental factors such as desiccation, salinity, UV radiation and temperature would have significant implications for the exploration of Martian habitability.

2.4 Life Under Multiple Extremes

It is clear when reviewing our current understanding of the adaptations to extreme conditions that there is considerable overlap in the mechanisms employed by microorganisms to deal with extremes individually. Indeed, our understanding of life at the very edges of these extreme conditions evidently must rely on the stresses imposed by more than one extreme pushing the boundaries of habitability even further. Yet research into the combined effect of multiple extremes on microbial life is surprisingly limited. This is even more surprising when considering very few organisms exist under the effect of a single physical or chemical extreme, instead life more commonly inhabits environments that are better defined by the effect of simultaneously occurring extremes. This absence of information is clearly outlined in a previous report by Harrison et al. (2013),

within which laboratory growth data of 67 prokaryotic strains was used to produce three-dimensional maps of the limits of microbial growth under the extremes of NaCl, temperature and pH (Figures 2.3 and 2.4). Though these maps are extremely useful in furthering our understanding of life in extreme conditions, they are lacking in data examining the combined effect of these stresses. For example, strains applied in this study such a *Pyroccocus yayanasii* is capable of growth between 80 and 108°C but requires high hydrostatic pressures to do so (20 to 120 MPa), and no growth occurs at any temperature below 20 MPa (Birrien et al., 2011). Conversely, *Colwellia piezophila*, grows optimally under 60 MPa pressure at the low temperatures of 4 and 10°C, with no growth under atmospheric pressure between 2 and 15°C or in the absence of NaCl (Nogi et al., 2004). The lack of information regarding the growth and physiological response of microbes to multiple extremes exposes a need for a more robust understanding of the response of organisms to concomitant stressors and a more detailed investigation of the physicochemical boundaries of life in the biosphere.

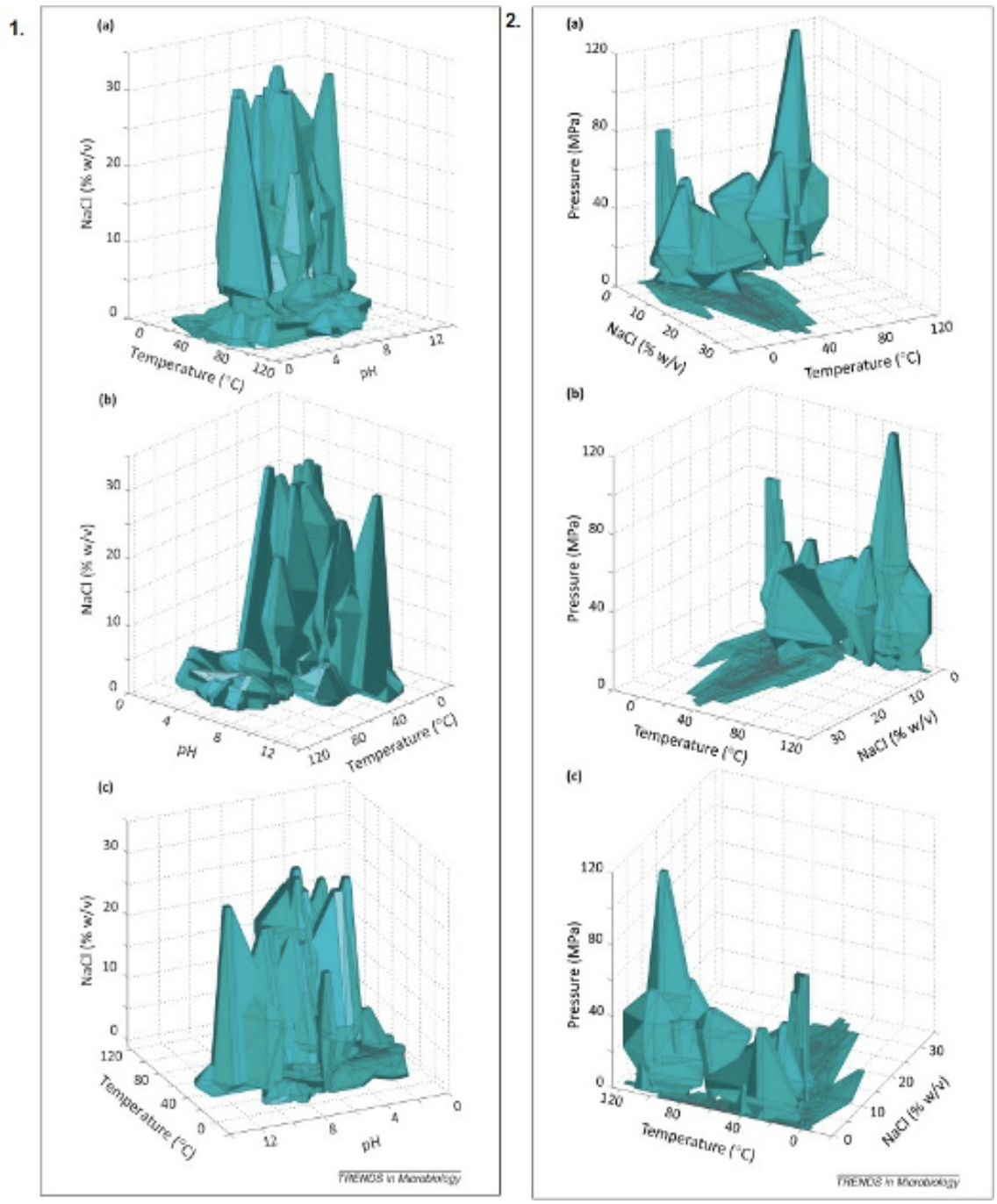

Figure (2.3) *The limits of terrestrial life determined as a function of 1: Temperature (°C), NaCl [% wt/vol] and pH, and 2: Temperature (°C), NaCl [% wt/vol] and pressure (MPa).*
These parameter spaces were estimated by applying known growth ranges of 67 prokaryotic strains. Each boundary space is presented from three angles [(a), (b) and (c)]. (Harrison et al., 2013).

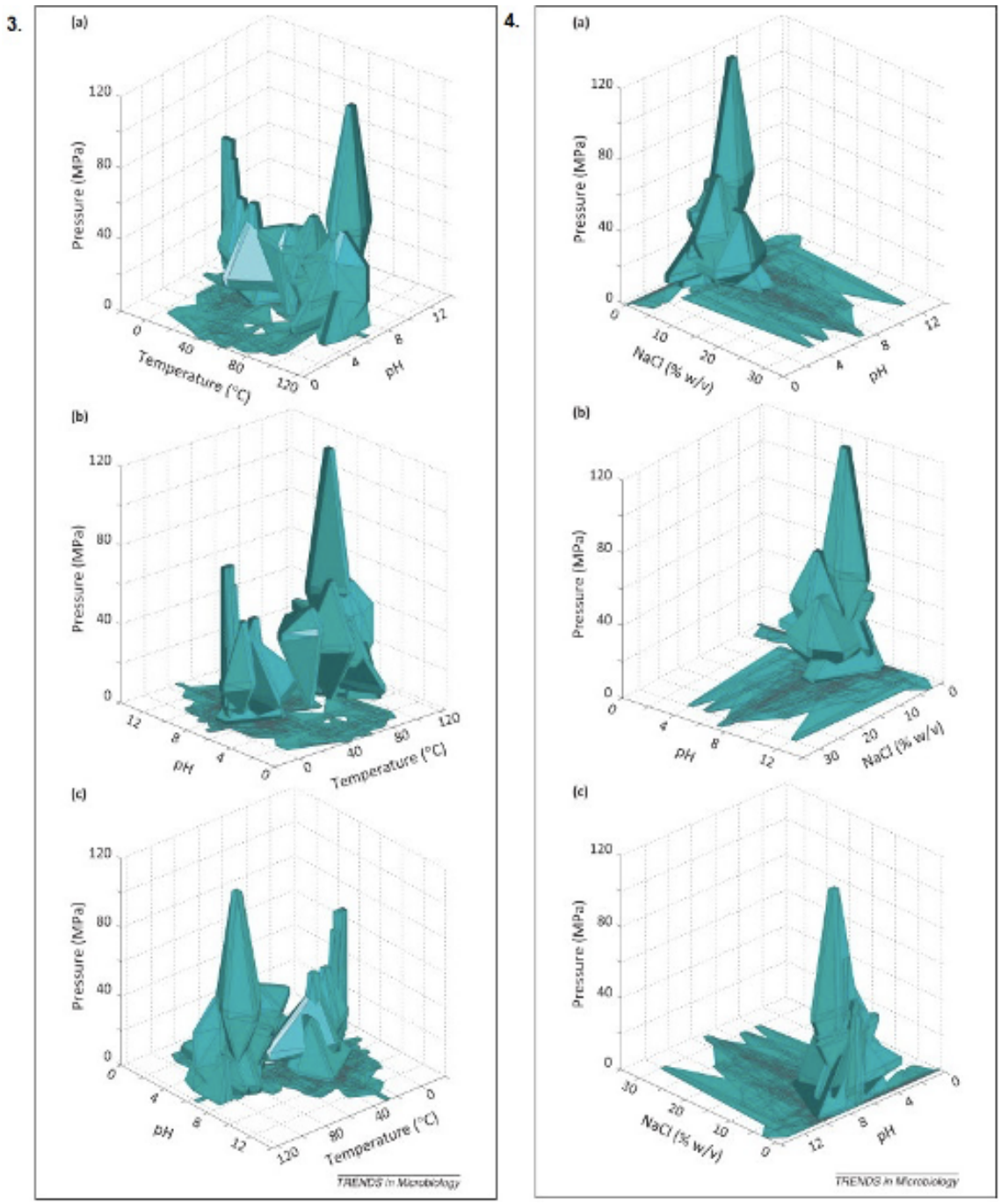

Figure (2.4) *The limits of terrestrial life determined as a function of 1: Temperature (°C), pH and pressure (MPa), and 2: NaCl [% wt/vol], pH and pressure (MPa).*
These parameter spaces were estimated by applying known growth ranges of 67 prokaryotic strains. Each boundary space is presented from three angles [(a), (b) and (c)]. (Harrison et al., 2013).

2.4.1 Adaptations to Multiple Extremes

Environments in which multiple physical and chemical extremes effect microbial growth are poorly understood despite multiple strains having been isolated from such habitats (Harrison et al., 2013). One important observation has been made in the sea-ice bacterium *Shewanella gelidimarina*, which when cultured at high salinity (NaCl) exhibits an increased temperature range for cellular division (Nichols et al., 2000). This maybe a result of a synergistic relationship between adaptations to cope with each extreme parameter conferring tolerance to the other, in this case the increase in membrane lipid packing and fatty acid content (Nichols et al., 2000; Russell and Fukunaga, 1990). Indeed, it is suggested that in Arctic marine bacteria have adaptations specific to dealing with extremes in cold temperatures and salinity that are encountered in the sea ice environment, but the link between the properties of these adaptations remains to be fully explored (Junge et al., 2004).

High NaCl habitats that are also subject to a combination of extremes in temperature and pH are not uncommon. Indeed, many high pH environments are additionally characterised by high saline conditions (Cioni et al., 1992; Czop et al., 2011; Zhilina et al., 1998), and a novel halophilic alkalithermophile strain, *Natranaerobius thermophiles*, has previously been isolated from soda lakes in Egypt that is capable of growth under a combination of high temperature, pH and NaCl (Mesbah et al., 2007). This particular strain is very intriguing as the mechanisms to maintain osmotic balance and pH homeostasis, which are intrinsically linked (Padan, 2001), are on the face of it, not compatible with high temperature adaptations that result in increased Na^+ and H^+ permeability through the membrane (Konings et al., 2002; Van De Vossenberg et al., 1999). Further to this, the same combination of extremes with acidic pH has also been shown to prove habitable in Lake Magic, Southern Western Australia, which is characterised by pH as low as 1.7, salinity up to 32% and experiences variations in temperature from 0 to 50°C (Benison et al., 2007; Bowen and Benison, 2009). This acid-saline lake plays host to bacteria, archaea and green micro-algae trapped in halite fluid inclusions (Conner and Benison, 2013). Though microbes capable of dealing with two extremes are well categorised, combinations of more than two extremes are not fully understood and the capacity for these organisms to cope with these particular extremes in combination outlines a necessity to further our understanding of combined stresses.

Microbial responses to temperature and pressure variations share distinct similarities. Alterations in membrane composition result in increased rigidity or fluidity under high and low temperatures respectively, and an increase in membrane fluidity is an adaptation shared by organisms capable of tolerating high pressures (Jaenicke and Sterner, 2006; Kato et al., 2008). It is unsurprising then to find that the simultaneous effect of pressure and temperature can push the boundaries of habitability under temperature extremes (Fichtel et al., 2015; Marteinsson et al., 1999; Takai et al., 2008). Additionally, mechanisms to deal with the effect of NaCl may prove synergistic under temperature and pressure extremes. The interaction of the mechanisms for NaCl concentration have previously been shown to increase pressure tolerance where the membrane increases stability through a decrease in unsaturated fatty acid content (Tanaka et al., 2001). This is surprising as to date, most known piezophiles have been isolated from low saline environments and halophiles from atmospheric, or near atmospheric, pressure conditions (Harrison et al., 2013), further adding to the need to assess the viability of life under a combination of these conditions. Sodium ion permeability is known to escalate with increased temperature (Figure 2.5), yet the additional effect of hydrostatic pressure on microbial membranes may increase the potential for life under a combination of these extremes.

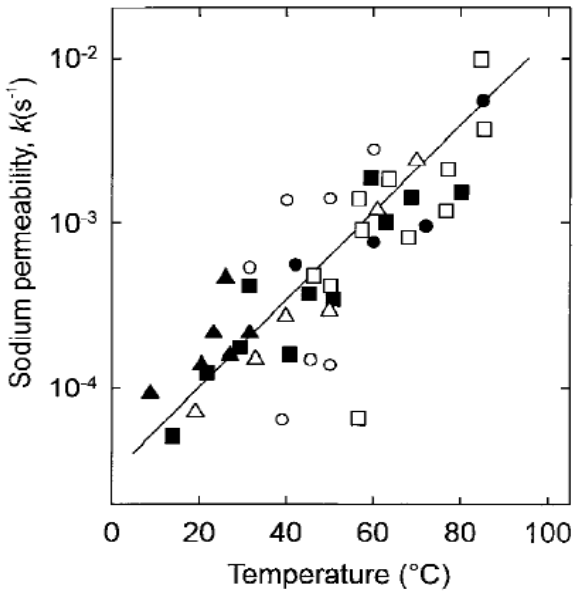

Figure (2.5) *Sodium ion permeability observed to increase with temperature in a number of bacterial and archaeal species.* P. immobilis *(▲),* M. barkeri *(△),* E. coli *(○),* B. strearothermophilus *(■),* T. martima *(●), and* S. acidocaldarius *(□) (Konings et al., 2002).*

2.5 The Cost of Living

Life on Earth has developed numerous ways to cope with the physical and chemical extremes that present themselves in the environment. However, for an organism to effectively operate the adaptive machinery to cope under these extremes, it must ultimately be able to meet the energy requirements necessary to cope with the environmental stresses imposed on it and support a functioning biochemistry (Cockell and Nixon, 2013; LaRowe and Amend, 2015). In theory, if an organisms energy resources are allocated to the adaptive mechanisms used to deal with one stress parameter, there will be fewer resources available to cope with other environmental stressors imposed (Bowers et al., 2009). The process of energy production in microbes consists of substrate-level or oxidative phosphorylation, the processes of which forms adenosine triphosphate (ATP), a universal molecular bearer of metabolic energy, and via energy transducing systems at the level of the cytoplasmic membrane which relies on transmembrane ion-gradients (Harrison et al., 2015a,b; Konings et al., 2002, 1994; Lane and Martin, 2012).

Energy yields in the form of ATP are known to vary considerably under aerobic and anaerobic conditions despite similarities in respiratory pathways, where we see the efficacy of aerobic respiration produces higher ATP yields than anaerobic respiration (King, 2005; Unden and Bongaerts, 1997). Energy transducing systems that rely on ion-gradients across the cytoplasmic membrane depend on membrane bioenergetics that in turn rely on impermeability to Na^+ and H^+ (Lane and Martin, 2012). As we have seen, membrane permeability is determined by multiple environmental factors such as pH and salinity, which may ultimately be a function of environmental temperature. This raises questions about the potential for organisms to deal with extremes of these conditions under aerobic and anaerobic conditions, and in particular: does the presence of oxygen release life from the constraints of energy limited anaerobic conditions? The latter has significant implications for whether the introduction of oxygen into the atmosphere during the Great Oxidation Event changed the limits to life on Earth.

2.5.1 Oxygen in Earth's Atmosphere

The first rise of atmospheric oxygen on Earth occurred around 2.4 billion years ago (known as The Great Oxidation Event) and then again approximately 700 million years ago (Catling and Claire, 2005). The rise in atmospheric oxygen was a product of oxygenic photosynthesis by cyanobacteria that occupied the planet in its largely anaerobic state (Anbar et al., 2007; Flannery and Walter, 2012; Kasting and Siefert, 2002; Lyons et al., 2014). Significant questions remain about where, and how, the metabolic process of oxygenic photosynthesis started, however it is clear that until the time at which atmospheric oxygen reached significant levels the development of complex multicellular organisms did not occur (Blankenship and Hartman, 1998; Buick, 2008; Catling et al., 2005; Schirrmeister et al., 2013). When molecular oxygen is found in significant concentrations, such as that introduced by oxygenic photosynthesis in Earths history, the use of oxygen in respiration by facilitative anaerobic organisms, which can grow in the presence or absence of molecular oxygen, is favourable over anaerobic respiration or fermentation (Richardson, 2000; Schmitz et al., 2013). When considering our understanding that aerobic strains exhibit a broader tolerance window to extreme conditions when compared with anaerobic strains (Harrison et al., 2015a), how this rise in atmospheric oxygen may have changed the capacity for life to cope with multiple extremes is a fundamental question when attempting to better understand life under concomitant stresses.

2.6 Addressing the Gap in Our Understanding: How Better to Define a Habitable Environment

This chapter has outlined the known boundaries for life under environmental extremes of salinity, pH, temperature and pressure and the strategies microbes have adapted to deal with these extremes, the importance of recognising most habitats on Earth are better defined by the effect of extremes conditions experienced in combination and the potential energetic limitations these combinations of extreme conditions may have on habitability.

As discussed, our current assessment of the habitability of a given environment

is solely based on an organisms ability to propagate within the boundaries of one extremes parameter experienced in isolation. Due to the complex interactions of multiple stress parameters that more commonly define a natural environment, to provide a deeper understanding of the net effect of these stresses is essential in determining the true limits of life, and thereby provide a more robust definition of habitability. Despite remarkable advances in defining the boundaries of habitability on Earth in recent decades and the applications to fields such as astrobiology, biotechnology and antibiotic resistance research (Harrison et al., 2017; Nichols et al., 2000; Pikuta et al., 2007; Rampelotto, 2010; Rothschild and Mancinelli, 2001; Tanaka et al., 2001), there is a paucity of information regarding the growth and physiological response of microbes to multiple extremes. This lack of information is what forms the foundation for the following chapters and the questions addressed are further outlined here:

Chapter 4: Does a combination of stresses of pH, salinity (NaCl [wt/vol]) and temperature restrict the growth limits of the model organism *H. hydrothermalis* more than these extremes do when experienced individually?
Answering this question would contribute towards a more robust definition of the true limits of life under multiple extremes by assessing the net effect of concomitant stresses on biomass accumulation of *H. hydrothermalis*.

Chapter 5: Does the presence of oxygen in the atmosphere release three facultative anaerobic strains (*Halomonas hydrothermalis*, *Escherichia coli* and *Carnobacterium pleistocenium*) from the energetic limitations of anaerobic respiration under multiple extremes?
Despite our understanding of the higher energy yields of aerobic respiration, little is known about the different collective impact of multiple extreme parameters on microbe-mediated reactions under aerobic and anaerobic conditions. To answer this question would contribute further to better defining the habitability of extreme environments and address questions regarding the introduction of oxygen into Earths atmosphere during The Great Oxidation Event, and whether this new energy source expanded the limits of life under multiple extremes.

Chapter 6: Does the physical environmental parameter of high pressure govern the capacity to deal with a combination of stresses of pH and salinity for the model organism *H. hydrothermalis*?
Pressure is a fundamental parameter of Earths biosphere, yet our understanding of the potential synergistic or antagonistic nature of environmental stresses under high pressure conditions is limited. Answering this question would contribute to

our understanding of life under multiple environmental stresses, particularly when taking into consideration the natural environment from which the model organism occupies.

Chapter 3

General Methodology

3.1 Introduction

This chapter will detail the general methodology used throughout this thesis, including microbial strains and culture techniques. The following are standard techniques employed in microbiology research. More specific details for methods and materials are presented in detail within the methodology section of each experimental chapter.

3.2 Bacterial Strains

This study used three different bacterial strains, which be briefly described in this section.

Halomonas hydrothermalis is a Gram-negative moderately halophilic, psychrotolerant marine bacterium isolated from low-temperature hydrothermal fluid at a depth of 2580 meters in the South Pacific Ocean (Kaye et al., 2004). It has previously been used to demonstrate the effect of multiple environmental extremes, including simulated hydrostatic pressure and total salt concentration, as well as nutrient availability and salinity (Harrison et al., 2015b; Kaye and Baross, 2004). It was employed in this study due to its ability to propagate under a wide range of temperatures and salinities (2 to 40°C and 0.5 to 22% NaCl (wt/vol)).

Escherichia coli is a non-pathogenic, Gram-negative, facultative anaerobe that is widely used as a model organism in laboratory-based studies. Under optimal conditions, *E. coli* has a doubling time of approximately 20 minutes and can be cultured in a wide variation of laboratory media with an optimal growth of 37°C, with growth up to 49°C (Fotadar et al., 2005).

Carnobacterium pleistocenium is a Gram positive, psychrotolerant facultative anaerobe isolated from Pleistocene ice in a permafrost tunnel in Fox, Alaska, USA. It was employed in this study due to its narrower growth parameters relative to *H. hydrothermalis* and *E. coli*, with a temperature range 0 to 28°C, a salinity range 0 to 5% NaCl (wt/vol) and a pH range 6.5 to 9.5 (Pikuta et al., 2005). This relatively narrow range of growth parameters for *C. pleistocenium* allows for an increased understanding of the net effect of multiple stresses over a more restricted parameter space.

3.2.1 Storage

For each bacterial strain used in this study, aliquots were prepared for long-term storage at -80°C allowing for use of isogenic bacteria throughout, making for a more reliable comparison of data over the period of the study. Stationary phase cultures were prepared for each bacterium with 25% (vol/vol) glycerol and aliquots were stored at -80°C for future use.

3.3 Microbial Culture Techniques

3.3.1 Aerobic Culture Techniques

All aerobic culture media were sterilised by autoclaving at 121°C for 20 minutes unless otherwise stated within the composition description, in which case media components were filter-sterilised using 0.22 μm syringe filters (Millex®) into pre-autoclaved culture vessels. Growth cultures were obtained by culturing of the respective bacterium within sterile 15 mL falcon tubes with loosed cap or within a 250 mL glass conical Erlenmeyer flask with foam bung (see individual methodologies). Cultures were grown at varying temperatures and revolutions per minute (RPM), for further details see experimental chapter methodologies.

All aerobic culturing was performed using standard aseptic microbial techniques using sterile equipment and conducted either within a laminar flow hood or the sterile field surrounding a Bunsen flame.

3.3.2 Anaerobic Culture Techniques

Anaerobic culturing was performed for experiments described in Chapter 5. Due to the nature of the experimental work carried out with facultative anaerobic strains in this thesis, the addition of reducing agents was strictly prohibited. Therefore, preliminary experimental work was carried out to determine the necessary time for nitrogen gas purging to achieve complete oxygen removal from the media. For information regarding these preliminary tests see Chapter 5.

Anaerobic media were prepared by purging prepared non-sterile solutions with nitrogen gas for a minimum of 3 hours per 500 mL in butyl rubber stopper sealed Duran bottles (see Chapter 5. for further details). To avoid the undesired injection potential contaminants the nitrogen gas purging was performed through a 0.22 μm syringe filter (Millex®) attached to the gassing needle. Due to the nature of this study (further detailed in Chapter 5) no additional reducing agents were used on the preparation of these media. Following gassing, the media was transferred to 50 mL serum bottles, sealed with butyl rubber stoppers and crimped then autoclaved at 121°C for 20 minutes. For media ingredients that were not to be autoclaved, the solutions were filter-sterilised using 0.22 μm syringe filters (Millex®) into pre-autoclaved culture vessels and nitrogen gas purging performed aseptically (see below). All anaerobic culture preparation was performed using sterile syringes and gassing needles, which were flushed with nitrogen gas for a minimum of 60-seconds to ensure no oxygen is introduced to the media or media component. Flame-sterilisation techniques were executed for the canula and the tops of the butyl rubber stoppers. All plastics used in the anaerobic component of this thesis (Falcon tubes, 96-well plates, cuvettes, pipettes, etc) were stored in the anaerobic chamber for a minimum period of 24-hours before use to ensure complete oxygen expulsion.

3.3.3 Quantification of Physico-chemical Parameters

3.3.4 Measurement of pH

Accurate measurements of media pH were critical for all data chapters in this thesis. pH was quantified using a Jenway 3510 pH meter calibrated to three points (pH 4, 7 and 10).

To ensure pH of the media was maintained during microbial growth and, in the case of anaerobically prepared media, nitrogen gassing, the addition of 10 mL/L of the buffer potassium morpholinopropane suifonate (MOPS) (pH adjusted to 8.2 with NaOH) was added to all media in this thesis. For each strain used in this thesis (see section 3.2), $\sim$ 50 mL cultures were prepared in individual 250 mL conical flasks topped with a foam bung. Following growth, $\sim$ 20 mL of culture were sterilised using through a 0.22 μm syringe filter (Millex®) and the growth media re-assessed for changes in pH. In all instances, no pH change was observed. Following nitrogen gassing, the pH of all anaerobic media was re-measured to establish changes in pH due to gassing. In all instances no pH change was observed. To ensure the desired pH value of each media did not vary as a result of temperature, following media preparation for each pH value assessed in this thesis approximately 50 ml of each media was aliquoted into individual 250 mL conical flasks topped with a foam bung. Flasks containing media were incubated at the temperatures assessed in each data chapter for a period of 7-days and media pH was re-measured. In all instances, pH was not found to change as a result of temperature variations.

3.3.5 Calculation of NaCl Concentrations

To ensure NaCl measurements were determined accurately, the NaCl concentrations of all media employed throughout each stage of the experimental methodology were calculated and accounted for when mixing to establish accurate salinity ranges were used. This was achieved by calculating the mass (mg) of NaCl in the total volume of starter culture for each strain. Following this, the mass of NaCl in for each concentration was calculated and the mass of the starter culture added to attain an accurate measurement of total NaCl in the final 200 μl growth culture within each well.

3.4 Data and Statistical Analyses

All collected data was first prepared using Microsoft Excel Version 16.41. Growth curves were visually observed and maximal OD_{600} values extracted using Microsoft Excel. All statistical analysis and figure production were carried out using RStudio v1.1.453 (RStudioTeam, 2018). Tables were created through imputing analysis data produced through RStudio into Microsoft Excel. Several R based statistical packages were used in the assessment of data throughout this thesis, details of which can be found in the methods for each data chapter.

Chapter 4

Simultaneously occurring extremes: charting the boundaries of habitability space on Earth

4.1 Introduction

The habitable parameter space of a given environment is defined by the multiple constraints that restrict an organisms ability to propagate, and therefore by the maximum range of environmental conditions that life is able to tolerate. The habitability space within which biological processes occur is determined by the physico-chemical conditions that restrict these processes, such as temperature, salinity, pH, pressure and other factors such as availability of liquid water, UV radiation and nutrient availability (Dartnell, 2011; Harrison et al., 2013; Merino et al., 2019; Pikuta et al., 2007; Rothschild and Mancinelli, 2001). Understanding how the chemical and physical boundaries of life shape the habitability space on Earth is important in many fields of research including astrobiology (Pikuta et al., 2007; Rampelotto, 2010), biotechnology (Arora and Panosyan, 2019; Nichols et al., 2000; Rothschild and Mancinelli, 2001; Tanaka et al., 2001) and in potential medical applications such as antibiotic resistance (Harrison et al., 2017). Advances have been made to further our understanding of the boundaries of habitability on Earth through the examination of individual extremes on the growth of microorganisms, however, these investigations frequently focus on an organisms capacity to cope with individual extremes in isolation and rarely

the simultaneously occurring stresses that more commonly define an extreme environment (Canganella and Wiegel, 2011; Cavicchioli, 2002; Dartnell, 2011; Freeman et al., 2016; Rothschild and Mancinelli, 2001). The lack of information regarding the growth and physiological response of microbes to concomitant extremes exposes a need for a more detailed understanding of the growth response of organisms under combined stressors. In this chapter, a systematic investigation of the effects of a combination of extremes in temperature, salinity and pH on the growth of the psychrotolerant, moderately halophilic hydrothermal vent bacterium *Halomonas hydrothermalis* was undertaken.

4.2 Background

The mechanisms of tolerance to extremes in salinity, pH and temperature extremes are well characterised (Baker-Austin and Dopson, 2007; Jaenicke and Sterner, 2006; Konings et al., 2002; Krulwich et al., 2011; Oren, 2006; Siliakus et al., 2017). Some laboratory studies have demonstrated the importance of understanding the effect of multiple stress parameters in defining habitability and have shown that in some instances adaptive mechanisms for one stress can be beneficial when dealing with another. For example, the barophilic archaeon *Thermococcus barophilus* was shown to have an increased growth rate at 85°C at higher pressure (40 MPa) compared with atmospheric pressure, as well as requiring 15 to 17.5 MPa for growth between 95°C and 100°C (Marteinsson et al., 1999), and the bacterium *Shewanella gelidimarina* displays an increased temperature range when cultured under high concentrations of NaCl (Nichols et al., 2000).

In theory, if a microorganisms resources are allocated to the adaptive mechanisms used to deal with one stress parameter (e.g. temperature) there will be fewer resources available to cope with other environmental stressors (Bowers et al., 2009). The composition of the cytoplasmic membrane of bacteria is an example of a common biochemical basis for adaptation to different extremes. Indeed, a crucial function of microbial cells is their ability to adapt rapidly to changes in the extracellular environment (for example increased NaCl concentrations) by adjusting the composition of their cytoplasmic membrane to maintain optimal intracellular conditions for energy transduction and metabolism (Aston and Peyton, 2007; Bremer and Krämer, 2019; Konings et al., 2002; Padan, 2001; Roberts, 2005). Common environmental parameters that negatively

affect membrane integrity are temperature, pH, and hydrostatic pressure, causing changes in the permeability and fluidity of the membrane (Konings et al., 2002; Nichols et al., 2000; Siliakus et al., 2017). Changes in membrane integrity may prove to be either a detrimental or an advantageous characteristic when attempting to cope with other environmental parameters such as high NaCl concentrations.

In natural environments, multiple stressors have been proposed to be the cause of low microbial diversity in some locations, such as in high temperature, low pH and high ion volcanic environments (Hynek et al., 2018). Despite the existing laboratory and field data, and our understanding that natural environments can be best characterised by the net effect of multiple environmental parameters, basic studies on the interplay between concomitant environmental extremes on microorganisms is limited. The work in this chapter tests the hypothesis that a combination of extreme environmental parameters of temperature, NaCl and pH restricts the growth limits of the model organism more than the individual extremes in isolation and quantifies these effects. These three factors were chosen because they are known to establish limits to life and have been the focus of a substantial number of studies individually (Aston and Peyton, 2007; Baker-Austin and Dopson, 2007; DasSarma and DasSarma, 2001; Jaenicke and Sterner, 2006; Krulwich et al., 2011; Méndez-García et al., 2015; Oren, 2006; Tolner et al., 1997).

4.3 Methods and materials

4.3.1 Strain

Halomonas hydrothermalis DSM-15725 was secured from the German collection of Microorganisms (DSMZ, Braunschweig, Germany). *H. hydrothermalis* exhibits cellular division between 2°C and 40°C (optimal growth at 30°C), NaCl concentrations between 0.5 and 22% [wt/vol](optimal range of 4 to 7% [wt/vol]), and pH between 5 and 12 (optimal range of 7 to 8) (Kaye et al., 2004). Owing to its ability to propagate under a broad range of temperature, salinity and pH ranges, *H. hydrothermalis* is a model organism for investigating the limits of life under a combination of extreme parameters. These three factors were selected due to their known limits to life and have been the focus of a significant number of

studies individually (Aston and Peyton, 2007; Baker-Austin and Dopson, 2007; DasSarma and DasSarma, 2001; Jaenicke and Sterner, 2006; Krulwich et al., 2011; Méndez-García et al., 2015; Oren, 2006; Tolner et al., 1997).

4.3.2 Growth assays

All experimental work in this chapter was performed aerobically. Growth cultures were obtained by culturing of the bacterium in minimal marine media (MMM) (Östling et al., 1991), altering the level of glucose to produce a MMM with 0.5 glucose composition, with 1.63% [wt/vol] NaCl (pH 8). Aliquots were prepared with 25% [vol/vol] glycerol, stored at -80°C and subsequently used to inoculate MMM agar (above recipe with the addition of 1.5% Agar Bacteriological No. 1) which were grown at 30°C for 48 hours and stored at 4°C until use. Starter cultures were prepared by transferring cells from agar plates to 5 mL MMM broth (1.63% NaCl [wt/vol] pH 8) in a loosely capped 15 mL tube (Sarstedt, Nmbrecht, Germany). The culture was grown for 24 hours in a shaking incubator (30°C, 120 rpm) and diluted with fresh MMM to a final cell density equivalent to an optical density at 600 nm (OD_{600}) of 0.2. Optical density measurements for growth assay starter cultures were obtained with a volume of 1 mL using the DR 5000 UV-Vis Spectrophotometer (Hach Company, Dsseldorf, Germany).

Growth assays were started by adding 10 L starter culture to fresh MMM broth at a range of salinities ($n = 21$) to a total volume of 200 μL in 96-well micro-plates with the accompanying lid (Greiner Bio-One, Frickenhausen, Germany). Twenty-one variations of MMM were used with 6.49%, 6.58%, 6.66%, 6.75%, 6.84%, 6.93%, 7.01%, 7.10%, 7.19%, 7.28%, 7.37%, 7.45%, 7.54%, 7.63%, 7.72%, 7.80%, 7.89%, 7.98%, 8.07%, 8.16% and 8.24% NaCl [wt/vol]. To alleviate pipetting error, aliquots for each salinity were prepared in 50 mL Falcon tubes by mixing MMM with 0 and 10% NaCl [wt/vol] and subsequent 96-well plate preparation performed. Three variations of culture media based on MMM under salinity ranges were prepared at pH values of 8, 7 and 6 using 1 M hydrochloric acid to lower pH. The pH was measured using a Jenway 3510 pH meter. Salinities and pH values were chosen for this study to cover values within the optimal parameters in addition to values supra-optimal (salinity) and sub-optimal (pH) to assess how minor variations outside of the boundary of optimal conditions affect growth.

All cultures were incubated at 30, 40, 41, 42, 43, 44 and 45°C.

These temperatures are reported to be the optimal (30°C) and supra-optimal (40°C) values for *H. hydrothermalis* under otherwise optimal conditions, with the additional exploration of further supra-optimal (41 to 45°C) temperatures. To ensure pH of the culture medium did not vary over the temperature range tested, 50 mL aliquots of each media pH variation were stored at each temperature used in this experiment for a period of 48 hours and the pH was measured.

The salinities, pH and temperatures used in this study were chosen as a method for exploring how minor variations in conditions both within and outside the optimal range for the model strain might act to limit, or broaden, the growth range when experienced in combination.

Cell density measurements were obtained every 10-minutes over a 24-hour period by OD_{600} measurement using a Synergy 2 microplate reader (BioTek Instruments Inc., Vermont, USA) shaken continuously at 1080 rpm. Each culture condition was observed in triplicate within a micro-plate and three micro-plates monitored per temperature and pH combination, providing $n = 9$ measurements for each culture variation. Medium only controls were included ($n = 6$) for each plate. Growth curves were plotted and visualized to determine the maximum OD_{600} value for each culture condition. As a consequence of very low maximal OD_{600} values attained under multiple extremes, it is necessary establish a cut of point for assumed propagation. This study assumes a maximal OD_{600} value >0.01 to be a result of microbial growth, any value obtained lower than this was deemed to be unreliable. Maximal optical density was chosen as a proxy for total cell biomass achieved in a given unit of time. OD_{600} readings can in principle be altered by cell size and shape or influenced by the number of live and dead cells, but for the purpose of this study it is considered be a sufficiently robust measure of growth for a single organism under the well-defined conditions of our experiment and this study defines 'no growth' as the condition where no observable growth by OD_{600} measurement was obtained during the 24-hour period.

4.3.3 Data analysis

For comparisons of maximal OD_{600} values under optimal temperature conditions (30°C) a two-way analysis of variance (ANOVA) was performed with salinity and pH as the factors. Maximum OD_{600} values under supra-optimal temperature conditions (40 to 45°C) were compared using three-way ANOVA with temperature, salinity and pH as the factors. To meet model assumptions,

visual inspection in combination with a Levenes test was performed to assess to the equality of variance. In order to alleviate heteroscedasticity of variance, measurements obtained for cells incubated under all culture conditions were Box-Cox transformed ($\lambda = 0.17$ and 0.07 for 30°C and 40 to 45°C, respectively).

For confirmation of where the differences occur between each combination of temperature, salinity and pH, the three-way ANOVA and Box-Cox transformed data were performed in conjunction with a Tukeys Honestly Significant Difference (HSD) test. All statistical analysis performed using RStudio v1.1.453 (RStudioTeam, 2018). Levenes test, Box-Cox transformation and Tukey HSD were competed using the 'car', 'MASS' and 'agricolae' package's, respectively.

4.4 Results

4.4.1 Effects of salinity and pH on maximal OD_{600} at optimal temperature

Under the optimal temperature condition of 30°C, *H. hydrothermalis* displayed cell division up to and including the highest concentration of NaCl in this study (8.24%) at all pH values tested, with notably smaller maximal OD_{600} values in cultures exposed to pH 6 than those at pH 7 and 8 (Figure 4.1). The reduction of maximal OD_{600} values for *H. hydrothermalis* with increasing concentrations of NaCl, or variation in pH (6 to 8) under optimal temperature conditions was demonstrated by a two-way ANOVA and Tukeys HSD. Significant effects of NaCl concentration and pH on maximal OD_{600} values were observed both individually and in combination (Table 4.1). Tukeys post hoc was used to compare the means of each culture condition (data not shown due to the high number of comparisons). Maximal OD_{600} values at pH 6 were significantly different to all values at pH 7 and 8. The most pronounced difference within pH 6 growth conditions were between salinities 6.75 and 8.07% NaCl [wt/vol]. For maximal OD_{600} values obtained within pH 7 and 8 growth conditions, the differences were most pronounced between pH 7 values at salinities <7.37% NaCl and pH 8 values at salinities >7.01% NaCl [wt/vol].

Table (4.1) *Two-Way ANOVA results for maximal OD_{600} Values at 30°C. These data correspond with the interactions between the factors salinity and pH. Cultures were incubated under a range of salinities (n = 21) and pH values of 8, 7 and 6. Data were Box-Cox transformed to alleviate heteroscedasticity of variance.*

Culture condition	**Df**	***F*-value**	***P*-value**
Salinity	20	36.386	<0.001
pH	2	4922.024	<0.001
Salinity$*pH$	40	5.979	<0.001

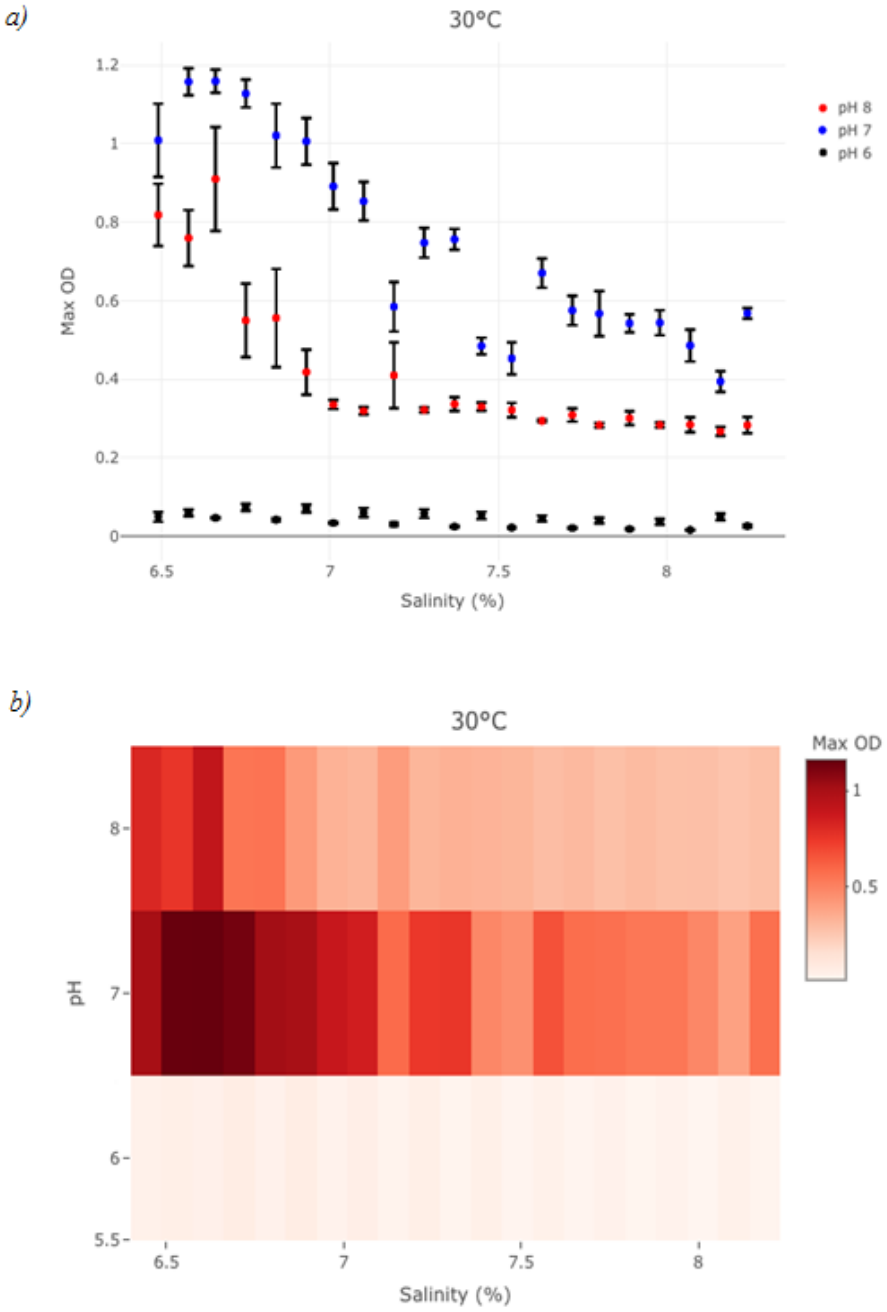

Figure (4.1) *Maximal OD_{600} values of H. hydrothermalis cultures (n = 9) under optimal temperature conditions (30°C) a) Maximal OD_{600} values for* H. hydrothermalis *cultures obtained under a variation of salinities (6.44 to 8.18%, n = 21) and pH (6 to 8, n = 3). Data shown as means and standard error of the mean. b) Heatmap displaying maximal OD_{600} values of* H. hydrothermalis *cultures (n = 9) at optimal temperature throughout each pH and salinity tested in this study.*

4.4.2 Effects of salinity and pH on maximal OD_{600} at supra-optimal temperatures

Under culture conditions at pH 8, temperature had a significant effect on maximal OD_{600} values at different concentrations of NaCl with *H. hydrothermalis* displaying cell division up to the highest salinity tested (8.24%) only at temperatures 40 and 41°C.

At 42°C, sensitivity to NaCl at pH 8 increased with growth ceasing at 7.01%, demonstrating a tolerance reduction of 1.23% NaCl with a temperaute increase of 1°C, and further sensitivity was observed at 43°C where growth was halted at 6.58% showing an additional tolerance reduction of 0.43% with a 1°C increase in temperature. Tolerance was reduced by a further 1.66% NaCl with a change of 2°C, where at temperatures of 44°C and 45°C, no cell division was observed (Figure 4.2 and 4.3).

Under culture conditions at pH 7, the pH of the media increased microbial sensitivity to NaCl with cell division ceasing at a concentration of 7.37% at 40°C, 6.75% at 41°C, demonstrating a tolerance difference of 0.62% with a 1°C temperature change, and 6.58% at 42°C further demonstrating difference of 0.17% with a 1°C temperature change. Similar to assays at pH 8, with culture conditions at pH 7 temperature had a significant effect on microbial propagation with no cell division at 43 to 45°C further decreasing salinity tolerance by 0.79% with a further 2°C temperature increase (Figure 4.2 and 4.3). Under culture conditions at pH 6 a combination of temperature and pH limit microbial propagation as no cell growth was observed at any NaCl concentration at supra-optimal temperatures (40 to 45°C) (Figure 4.2).

The reduction of maximal OD_{600} values of *H. hydrothermalis* under the combinations of the three different extremes, supra-optimal temperatures, NaCl concentration and pH (6 to 8) was further demonstrated by three-way ANOVA and Tukeys HSD test comparing maximal OD_{600} values. Significant effects of temperature, NaCl concentration and pH were observed both individually and in combination (Table 4.2). Tukeys post hoc was used to compare the means of each culture condition (data not shown due to the high number of comparisons). Under culture conditions at pH 8, at 40°C the most pronounced differences in maximal OD_{600} values were between 6.49 to 6.75% and 7.37 to 8.24% NaCl [wt/vol] (excluding 7.45%), showing that maximal OD_{600} values obtained between

6.49 to 6.75% NaCl were significantly different to those obtained between 7.37 to 8.24% NaCl [wt/vol]. Differences were observed at 41°C where salinity ranges between 6.49 to 6.75% NaCl were significantly different to those obtained at 7.98% NaCl and at 42°C where maximal OD_{600} values obtained between 6.49 to 6.58% NaCl were significantly different to those between 7.10 to 8.24% NaCl [wt/vol]. There were shown to be no significant differences between maximal OD_{600} values within temperatures 43 to 45°C. Under culture conditions at pH 7: at 40°C the differences lie between 6.49 and 7.45 to 7.89% NaCl [wt/vol] (excluding 7.89%). Owing to low maximal OD_{600} values obtained at pH 7, at 41 to 45°C, despite growth occurring at lower salinities, post-hoc analysis shows there to be no significant differences. Differences in maximal OD_{600} values between pH culture conditions were pH 8 salinities 6.49 and 6.75% at 40C and 7.19% NaCl [wt/vol] at 40°CC under pH 7 culture conditions.

Table (4.2) *Three-Way ANOVA results for maximal OD_{600} Values at 40-45°C. These data correspond with the interactions between the factors temperature, salinity and pH. Cultures were incubated under a range of temperatures (n = 5), salinities (n = 21) and pH values of 8, 7 and 6. Data were Box-Cox transformed to alleviate heteroscedasticity of variance.*

Culture condition	**Df**	***F*-value**	***P*-value**
Temperature	5	1167.652	<0.001
Salinity	20	64.279	<0.001
pH	2	1829.503	<0.001
Temperature$*Salinity$	100	10.255	<0.001
Temperature$*pH$	10	296.030	<0.001
Salinity$*pH$	40	19.358	<0.001
Temperature$*Salinity*pH$	200	3.103	<0.001

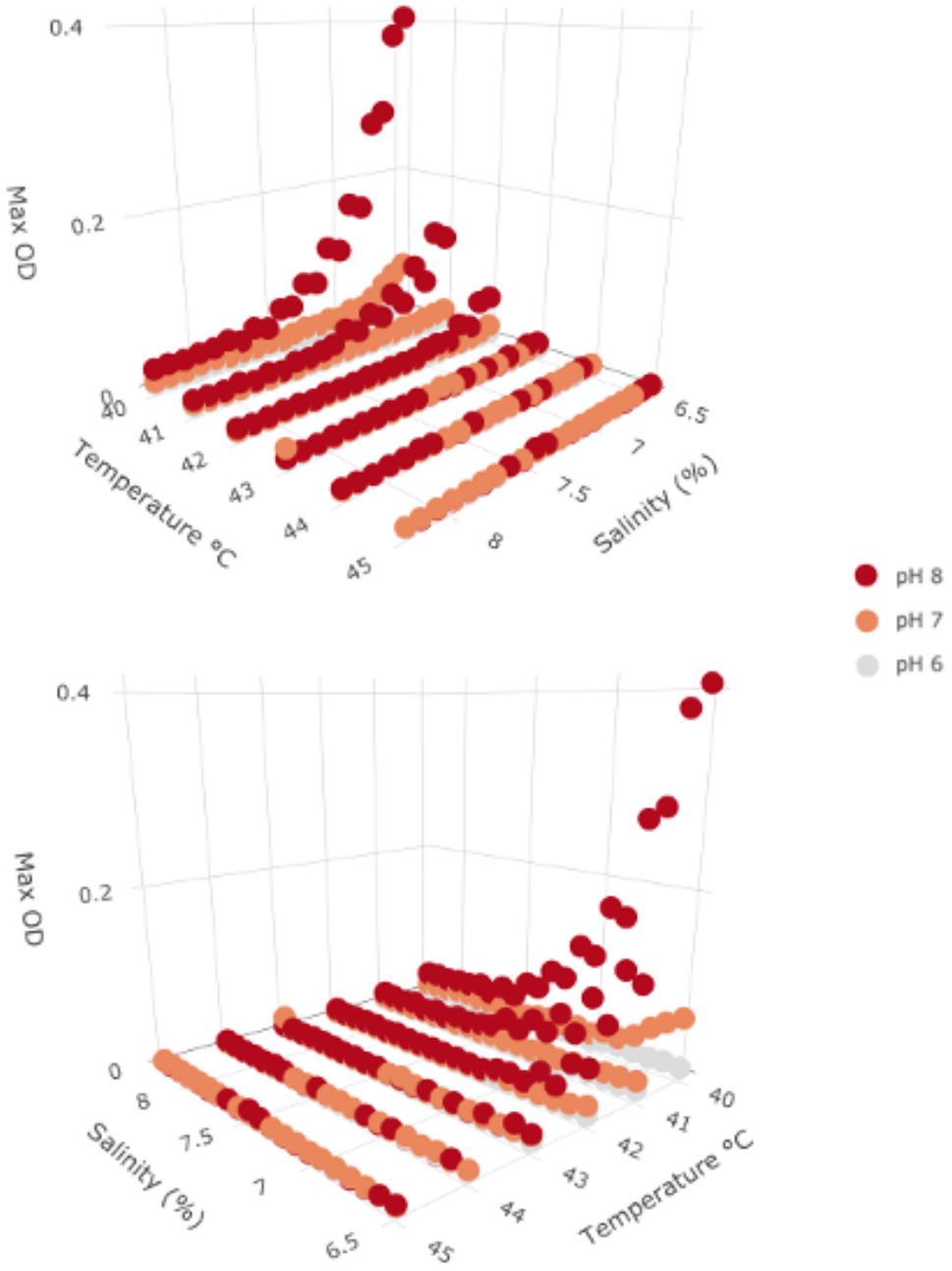

Figure (4.2) *Maximal OD_{600} values of* H. hydrothermalis *cultures ($n = 9$) at supra-optimal temperatures. Values were obtained for cultures grown under a temperature range of 40 to 45°C ($n = 5$), variations in salinity (6.44 to 8.18%, $n = 21$) and different pH values (6 to 8, $n = 3$). Data are provided from two arbitrarily selected angles to present a complete picture of the boundary space*

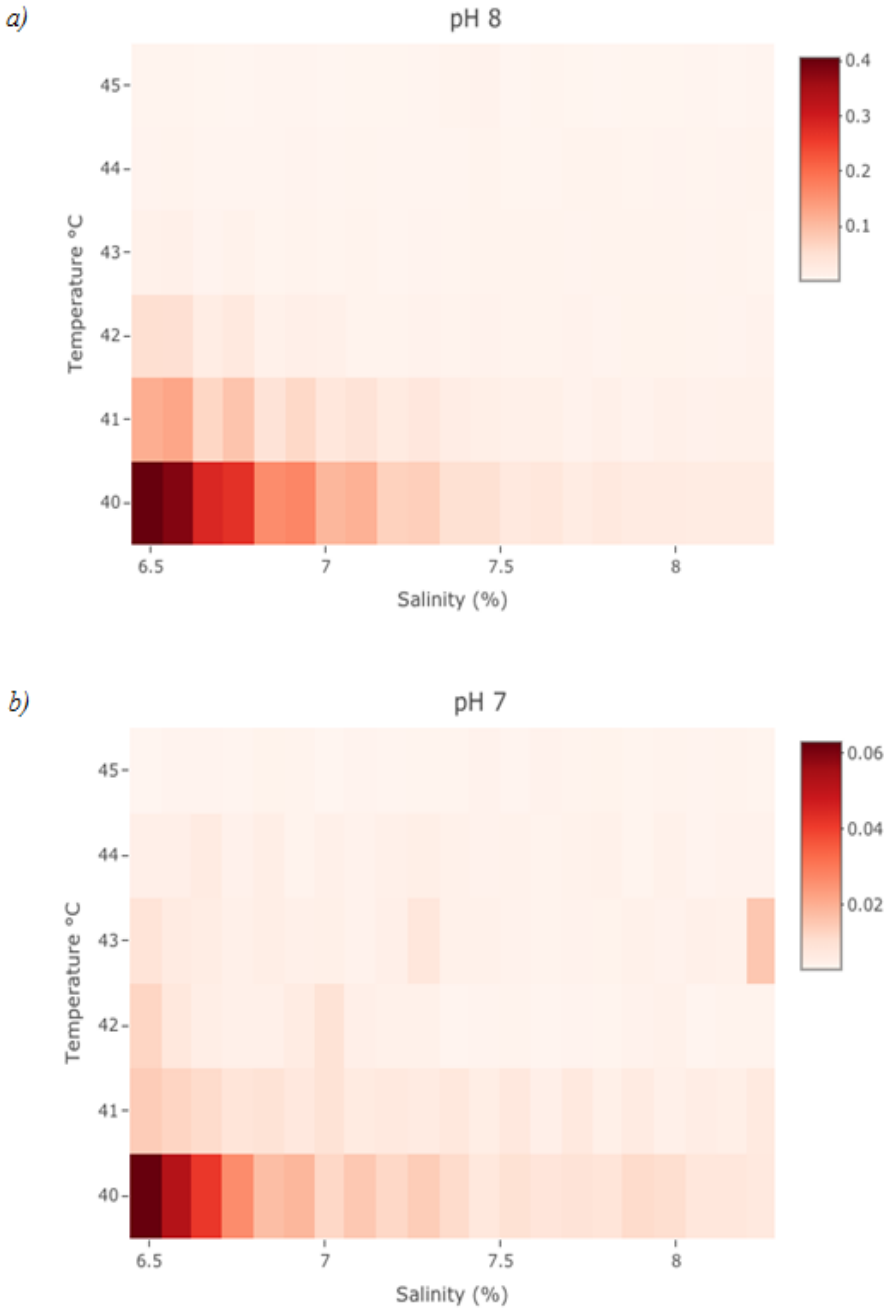

Figure (4.3) *Heatmaps displaying maximal OD_{600} values of* H. hydrothermalis *cultures (n = 9) under a range of temperatures (40 to 45°C, n = 5) and salinities (6.44 to 8.18%, n = 21) at pH 8 (a) and pH 7 (b). Data omitted for pH 6 as no maximal OD_{600} value was above 0.01*

4.5 Discussion

Investigating the response of microbial growth to single and combined environmental stresses is essential to further our understanding of the limits to life. Despite a multitude of studies examining the effects of individual environmental extremes on a large number of organisms (Aston and Peyton, 2007; DasSarma and DasSarma, 2001; Jaenicke and Sterner, 2006; Konings et al., 2002; Krulwich et al., 2011; Méndez-García et al., 2015; Merino et al., 2019; Mesbah and Wiegel, 2012; Oren, 2006; Tolner et al., 1997) which are often used on their own to define the limits to life, our understanding of the effect of multiple environmental stresses is surprisingly limited.

It is thought that the presence of multiple extremes in natural environments, such as high temperatures, low pH and high ionic concentrations in volcanic lakes (Hynek et al., 2018) combine to explain the low diversity of life in these environments, yet few laboratory studies have been done to systemically explore the interactions of three or more stressors on the limits of microbial growth. A previous report by Harrison et al. (2013) examined the laboratory growth data of 67 prokaryotic strains to produce three-dimensional maps of the limits of microbial growth under the extremes of NaCl, temperature and pH but the lack of data on the combined effects of these stresses meant that these maps represent the limits of life only under single imposed stresses. To improve our ability to map the true limits to microbial growth, these maps should be constructed using growth data in which multiple realistic extremes are combined.

Furthermore, it might hypothesised that microbial extremes could imply that the physico-chemical limits for the biosphere as a whole may be much smaller than those defined by growth under single extreme would suggest. Few natural environments on the planet exhibit only one extreme and thus attempts to define the biospheres boundary space based on the artificiality of single extremes may mislead our view of the limits to life. By contrast, in some instances, if one extreme mitigates another extreme (Nichols et al., 2000) we may underestimate the capacities of the biosphere. With this in mind, the final achievable cell biomass of the polyextremotolerant bacterium *H. hydrothermalis* (Kaye et al., 2004) was examined under multiple environmental stress parameters. This was achieved through a series of microbial growth assays to determine the individual effects of stresses, where growth under varying salinities can be measured at optimal temperature and pH and growth under varying pH can be measured

under varying salinties, and the combined effects of supra-optimal temperature, varying salinity (NaCl) and pH at the extremes of growth for *H. hydrothermalis*.

When cultured under optimal temperature conditions, the strain *H. hydrothermalis* was able to multiply under a broad range of salinities and displayed decreased maximal OD_{600} values with increasing salinity (NaCl) (Figure 4.1). It was found that one pH unit departures from neutral pH resulted in changes in tolerance to concentrations of NaCl. At pH 8 decreased maximal OD_{600} values were observed at lower concentrations of NaCl than those observed at pH 7. The pH was found to exert a stronger effect than NaCl on growth at optimal temperatures. This may be caused by the effects of pH on the maintenance of intra-cellular homeostasis resulting in the impairment of essential cellular processes (Baker-Austin and Dopson, 2007). pH is known to be the dominant factor in determining microbial community composition and abundance (Merino et al., 2019) and adaptations to both alkaline and acidic pH rely on maintaining intracellular neutrality through proton uptake or efflux over the cell membrane (Baker-Austin and Dopson, 2007; Krulwich et al., 2011).

When cultured at pH 6, the effect of salinity on growth was negligible, as the maximal OD_{600} values obtained demonstrate that the pH completely dominated growth. We suggest that at pH 6, the limitation to growth is such that additional limitations to growth imposed by NaCl have no additional discernible effect on cell physiology. Under optimal temperature conditions, maximal OD_{600} values were observed to be higher under culture conditions at neutral pH 7 then at the pH 8 and pH 6, with pH 6 values being lower than both pH 7 and 8. These data demonstrate the multiplicative effect of pH and NaCl at the optimal temperature for this organism (30°C). These findings show that, under optimal conditions for a single parameter, the addition of multiple environmental stresses of NaCl (%) and pH act to limit microbial growth. These data show that the assessment of the habitability of natural environments should go beyond consideration of an individual extreme to encompass the multiple physico-chemical properties as a whole.

The cultures used for this study were able to multiply over a range of supra-optimal temperatures, including those exceeding the previously published temperature limit for this organism (40°C) (Kaye et al., 2004). Similarly to the observations at optimal temperatures, at supra-optimal temperatures it is observed that the growth limits under NaCl were reduced by both pH and temperature experienced individually and in combination, where growth is limited

within the optimal pH range for *H. hydrothermalis* (7 to 8) with increasing supra-optimal temperatures, and growth is further limited under mildly acidic pH 6.

While the specific adaptive mechanisms of *H. hydrothermalis* were not investigated in this study, adaptations to the stresses imposed in this analysis are well characterised. One potential explanation for these data could be changes in membranes. For example, supra-optimal temperature adaptations in mesophilic bacteria can involve structural modification of the membrane such as adopting a more fluid and permeable structure by increasing the chain length of lipid acyl chains and the degree of saturation (Konings et al., 2002; Siliakus et al., 2017). This modification increases the ion-permeability of the membrane, potentially directly influencing the mechanisms controlling extremes of pH or NaCl (%) (Konings et al., 2002). An increase in permeability of the *H. hydrothermalis* membrane at supra-optimal temperatures could explain the increase in the sensitivity to NaCl at increasing temperatures and the interaction between these two extremes.

It was also observed that at any given temperature, pH dominated the final biomass over NaCl, with lowest final biomass observed at pH 6 compared to pH 7 and 8, with pH 8 being the optimal pH. The challenge of maintaining near neutral intracellular pH in bacteria requires the active pumping of protons out of the cell across the cytoplasmic membrane, the composition of which is one of the determining factors in surviving extremes of pH (Krulwich et al., 2011; Siliakus et al., 2017). One explanation for the observations of this study would be that at pH 6 and 7 cells were unable to maintain regulation against the high extracellular proton concentrations compared to their optimum at pH 8, slowing growth regardless of supra-optimal temperature or NaCl concentration.

Mildly acid pH conditions could also be affecting cell metabolism. Maintaining a pH gradient across the cell membrane requires energy and the availability of that energy may be significantly limited by the impact of multiple stresses interfering with a cell's capacity to transport solutes across the membrane (Jin and Kirk, 2018; Padan, 2001). Cell metabolisms are known to be affected by a broad spectrum of variables including temperature, salinity, pH and nutrient availability (Jin and Kirk, 2018). Indeed, microbial sensitivity to environmental extremes has previously been shown to be impacted by one particular extreme parameter in *H. hydrothermalis*, where iron (Fe^{3+}) starvation was shown to determine susceptibility to variations in temperature and salinity (Harrison et al., 2015b). pH is known to induce significant metabolic responses in microbial

communities and is known to be the principal determining control (Jin and Kirk, 2018). Therefore, stress induced by pH may influence the capacity to adapt to supra-optimal temperatures/NaCl and vice-versa, explaining the significant interactions between all three factors.

These data have shown that to assess the habitability, or capacity of a given environment to support microbial growth and reproduction, based solely on an organisms ability to propagate within the boundaries of one extreme parameter is to do so without taking into account the combined effect of concomitant stresses within that habitat. Indeed, habitats that fall within the boundary space of habitability as predicted by a single stress may be so influenced by additional parameters that although each single stress might predict an organism can propagate there, the combined factors mean that the environment lies outside of the known habitable conditions for life.

These results have application in astrobiology. For example, when considering the theoretical habitability of planetary bodies such as Mars, Enceladus and Europa one must consider the net effect of all environmental parameters imposed on the respective habitat. For example, the presence of brines that require high levels of salts such as sulfate, iron and perchlorates to remain liquid on the Martian surface have been proposed as locations to test for the presence of habitable conditions on Mars (Stevens et al., 2019). When considering the habitability of such brines it is imperative to examine the combined effect of other conditions known to influence those habitats such as temperature (Cavicchioli, 2002), pH (Fairén, 2010), desiccation (Stevens et al., 2019), water activity (Javier Martín-Torres et al., 2015), atmospheric pressure and UV radiation (Cockell, 2014), and how these environmental conditions have changed over time (Ehlmann et al., 2016). As on Earth, the assessment of any one of these parameters as lying within the growth and reproductive capacities of known organisms does not mean that the environment, when all of its stresses are imposed on an organism, is habitable.

4.5.1 Limitations

Despite the significant data collected in this chapter regarding the impact of multiple extremes on the limit of life, there are limitations to take into consideration. Natural environments can be further characterised by a multitude of conditions such as UV radiation, heavy metal concentration and bioavailability,

oxygen concentration, pressure and water availability that are not explored in this study. This is a limitation of this approach as one should consider the total net value of extreme parameters imposed on an organism in the natural environment to gain a more thorough understanding of the impact of multiple extreme conditions. Despite this limitation, it is clear from these data that multiple extremes significantly alter the capacity to deal with individual stresses that are permissible under otherwise optimal conditions.

4.5.2 Future Work

The data reported in this chapter focuses on the impact of multiple extremes on the propagation of the psychrotolerant, moderately halophilic marine bacterium *Halomonas hydrothermalis*. Future experiments using a diverse selection of both mesophilic, extremophilic and extremotolerant organisms could provide additional data on the impact of multiple environmental stresses on microbial propagation and viability. This also requires the growth response of the selected model organisms to be evaluated under the influence of previously unexplored concomitant environmental stresses. Further research into the adaptive mechanisms that contribute to the antagonistic or synergistic relationship between environmental factors can shed light on the overlapping characteristics of microbial adaptations that form the complex interplay between multiple physicochemical parameters. This could be achieved through various genomic, proteomic and metabolomic methods to assess gene expression, quantification of proteins under specified conditions and metabolic response to multiple abiotic influences.

4.5.3 Concluding remarks

Extremes in NaCl concentration, pH and temperature are known to significantly impact microbial propagation when experienced in isolation. However, this work demonstrated that, when these extremes are combined, they restrict the growth limits of the model organism more than when these individual extremes are experienced in isolation. Examples of this can be seen in figure 4.1a. The optimal NaCl (%) range for the model organism is between 4 and 7% [wt/vol], and growth is shown to differ between one pH unit departure from neutral pH 7 within this range thereby providing an account of the individual

effect of pH change on growth, and further to this, with increasing NaCl (%) growth can be seen to be limited by NaCl under differing pH culture conditions. Additionally, supra-optimal temperatures act to further limit growth with increasing NaCl concentration where we see a combination of the three stresses (4.2).These findings show the need for a more robust understanding of the limits of life under combinations of extreme conditions that more commonly define natural environments to allow for more accurate predictions on the boundaries of habitability.

Chapter 5

Energetic limitations of life under multiple extremes

5.1 Introduction

If an organism's energy resources are allocated to the adaptive mechanisms used to deal with one stress parameter, there will in theory be fewer resources available to cope with other environmental stressors imposed (Bowers et al., 2009). The biological demand for energy, the potential for meeting that demand and the energy expenditure when dealing with extremes further defines the habitability of natural environments (Hoehler, 2007). When considering differentially respiring organisms and their respective theoretical energy yields, the potential for life to exploit newly available energy sources, such as the rise in atmospheric oxygen from the Great Oxidation Event (Catling and Claire, 2005), could dramatically change lifes ability to cope with multiple extremes due to increased or decreased metabolic energy yields. The introduction of significant levels of atmospheric oxygen in Earths history happened around 2.4 billion years ago and then again approximately 700 million years ago, allowing for the development of complex multicellular life over a large expanse of time (Catling et al., 2005). How this newly available energy source changed the capacity of microorganisms to deal with multiple extreme conditions remains unknown. With this in mind, this study aims to address the question does the presence of oxygen release life from the constraints of low energy anaerobic conditions and change the limits of life under multiple extremes?.

This chapter explores the effect of multiple stresses of salinity, pH and temperature on microbial propagation using three facultative anaerobic strains (*Halomonas hydrothermalis*, *Escherichia coli* and *Carnobacterium pleistocenium*). By culturing these strains under combinations of these stress parameters both aerobically and anaerobically, the aim is to provide a better understanding of the energetic limits to life by assessing the maximal growth values attained under different modes of respiration. In this chapter, growth data collected under aerobic conditions is directly compared with results collected under anaerobic conditions using the same physicochemical stress parameters for each condition. Though there is a plethora of research identifying the boundaries for growth under these stresses individually for both aerobic and anaerobic strains (see Harrison et al. (2015a)), there is limited research assessing the effect of these stresses in combination. The results from this chapter further outlines the need to improve our understanding of the effect of concomitant extremes when determining the potential habitability of a given environment and outlines the importance of taking into consideration the differential energy requirements of organisms and the impact of environmental extremes on cellular energetics.

5.2 Background

5.2.1 Bioenergetics

For an organism to propagate in a given environment it must be able to meet the energy requirements necessary to cope with the environmental stresses imposed on it and support a functioning biochemistry (Cockell and Nixon, 2013; Hernandez and Newman, 2001; LaRowe and Amend, 2015). Microbial cells generate metabolic energy through substrate-level or oxidative phosphorylation, the process of which forms adenosine triphosphate (ATP), and via energy transducing systems at the level of the cytoplasmic membrane (Harrison et al., 2015a; Konings et al., 2002). ATP yields vary considerably under aerobic and anaerobic respiration (Unden and Bongaerts, 1997) and despite the similarities between respiratory pathways, the efficacy of anaerobic respiration is known to produce lower ATP and growth yields than aerobic respiration (King, 2005). Additionally, energy transduction in the cytoplasmic membrane is driven by environmental factors such as pH and salinity, which may ultimately be a function of environmental temperature (Booth, 1985; Konings et al., 2002;

Russell and Fukunaga, 1990). Microbial metabolisms are controlled by broad range of environmental factors including salinity, temperature, pH and nutrient availability (Amend et al., 2013; Harrison et al., 2015b; Lennon and Jones, 2011). This work aims to test the hypothesis that aerobic respiration allows for a broader range of cell division under extreme conditions than aerobic respiration due to higher energy yields where one would expect to measure higher biomass accumulation in aerobic cultures when compared with anaerobic cultures.

5.2.2 Energy and Extremes

Environmental stresses, such as variations in salinity, pH and temperature, can have notable impact on microbe-mediated reactions particularly when multiple stresses are experienced collectively (Harrison et al., 2015a). The three stresses of salinity (NaCl), pH and temperature are common in natural environments, the necessary adaptations to deal with them are well characterised (Baker-Austin and Dopson, 2007; Jaenicke and Sterner, 2006; Konings et al., 2002; Krulwich et al., 2011; Oren, 2006; Siliakus et al., 2017), and their known limits to life have been the focus of a considerable number of studies (Aston and Peyton, 2007; Baker-Austin and Dopson, 2007; DasSarma and DasSarma, 2001; Jaenicke and Sterner, 2006; Krulwich et al., 2011; Méndez-García et al., 2015; Oren, 2006; Tolner et al., 1997). Additionally, the impact of these environmental stresses on energy demand have been defined in detailed studies [see Oren (1999) for salinity, Krulwich (2006) and Krulwich et al. (2011) for pH, and Jaenicke and Sterner (2006) for temperature], which are briefly charactersed here:

NaCl

When dealing with high concentrations of NaCl in the environment, microorganisms use two strategies in order to maintain osmotic balance between their cytoplasm and the environment (Oren, 1999; Vreeland, 1987), the mechanisms of which are characterised as the salt-in strategy and the low salt-in (or compatible-solute) strategy (Oren, 2006). The salt-in strategy, involves maintaining high concentrations of potassium ions within the cell and extensive adaptations of the intracellular systems are essential for this strategy, which can only be achieved in a long and complex evolutionary process (Oren, 2006, 2008). As osmolality in hypersaline environments can be detrimental to cells, this method prevents

loss of water to the external medium through osmotic balance (DasSarma and DasSarma, 2001). The low salt-in, which is the favoured option for the majority of known microbes, is employed despite increases in energy demand, which may be due to this method requiring no specific adaptation to intercellular systems (Mesbah and Wiegel, 2008; Oren, 1999). This method involves the maintenance of the cytoplasm at a much lower NaCl concentration than the extracellular environment and requires the outward movement of Na^+ and intracellular accumulation of organic solutes across the cytoplasmic membrane, which is crucial to osmotic balance and pH regulation (Dartnell, 2011; Oren, 2006). A variety of bacterial species, including *Halomonas* strains, are known to oxidize organic compounds while they accumulate organic solutes (low salt-in strategy) and aerobic heterotrophic metabolism is achievable up to saturation of NaCl (350 g/L) (Oren, 2011). The fundamental energy source for expulsion of Na^+ and the acquisition of organic solutes by the majority of microorganisms is derived from either the respiratory electron transport or by using ATP established during oxidative phosphorylation (Oren, 1999).

pH

Variations of environmental pH from neutral pH 7 can be damaging to cellular biochemistry (Krulwich et al., 2011) and regulating intracellular homeostasis by the pumping of protons across the cell membrane requires a continuous consumption of energy (Booth, 1985; Konings et al., 2002; Siliakus et al., 2017). pH is described as the chemical activity of protons that are key in microbial redox reactions, which in turn drives ATP synthesis and ultimately energy availability (Siliakus et al., 2017). When environmental pH differs from an organisms optimal pH range (usually within a range of 3 to 4 pH units), microbial growth rates can decrease by as much as 50% with a pH change of only one unit (Rosso et al., 1995). Through directly affecting metabolism, environmental pH can shape microbial community composition. This is because pH can regulate respiration and growth, where respiration reactions become unfavourable due to their dependency on the energy available from redox reactions which a change with pH (Bethke et al., 2011; Jin and Bethke, 2002, 2003). Therefore, by inhibiting microbial respiration, and thereby controlling the amount of free energy available for use, environmental pH can shape community structure, which in turn can be determined by an organisms ability to cope with stresses of pH.

Temperature

The impact of temperature on microbial life is established by the fundamental principles of thermodynamics, which are not discussed in detail here (see Jaenicke and Sterner (2006)). In essence, increasing temperature results in a decrease in complexity and increases the rate at which reactions occur; therefore, metabolic reactions are fundamentally governed by temperature (Gillooly et al., 2001). This effect of temperature on microorganisms is directly applicable as it is not possible on a microscopic scale to maintain a physiological temperature difference to that imposed by the environment, and therefore the effects of temperature directly impact cellular processes and biochemistry (Hoehler, 2007). Microbial adaptations to high temperatures involve alterations to the lipid composition of the cellular membrane, which changes proton permeability through increased motion of the lipid molecules (Konings et al., 2002). Aerobic thermophiles can indemnify this increased proton permeability by increasing their respiration rate (Konings et al., 2002), however differences in energy yields from aerobic and anaerobic respiration may influence microbial survivability under sub- or supra-optimal temperature conditions.

Understanding the complex interplay of multiple stresses under different energy-yielding metabolic processes provides further insight into characterising the net effect of concomitant extremes on habitability. Exploring the impact of the combined effect of salinity, pH and temperature under aerobic and anaerobic conditions using three facultative anaerobic strains is the primary focus of the work presented in this chapter.

5.3 Methods and Materials

5.3.1 Aerobic and Anaerobic Culture Techniques

To establish an efficient method for quantifying the effect of oxygen limitation under a combination of environmental extremes it was necessary to establish a protocol that limits the impact of anaerobic media additives on microbial propagation. Therefore, anaerobic media for this study was prepared without the addition of any oxygen reducing agents to maintain media consistency throughout both the aerobic and anaerobic components of the analysis. Preliminary tests

were performed to assess the efficacy of both the nitrogen gassing of the media without the ensuing addition of a reducing agent and the adhesive sheets used for sealing the plate after inoculation.

For the preliminary tests, each media used in this study was prepared anaerobically (see Chapter 3) with the addition a 0.1% resazurin solution as an oxygen indicator. The resazurin solution was added into the media by pipetting 0.1 ml/L or until medium achieves the desired true blue colour. Following this, media was nitrogen purged until the colour changed to pink, indicating reduced oxygen. In the first instance, once the pink colour was achieved (approx. 1 hour) and the media was removed and autoclaved. Following autoclave sterilisation, in all instances the media remained pink in colour indicating the partial presence of oxygen. The process was repeated until the required time to achieve complete oxygen removal, as indicated by a colourless resazurin after autoclaving, was achieved. In all cases, this was accomplished after approximately 3 hours of nitrogen gassing at 0.5 bar pressure for 500 ml of media.

Following this, a 96-well plate (Greiner Bio-One, Frickenhausen, Germany) was prepared by aliquoting 200 μL of anaerobically prepared media into each well and sealing with a non-gas permeable adhesive PCR plate seal (Thermo-Fisher Scientific) within the anaerobic chamber. Sealed plates were then removed from the chamber and left at room temperature and at the maximum temperature tested in this study for the respective organism (see Section 4.3.4), to ensure no oxygen exposure to the media. This test was performed for each media used in this study at a concentration of 0 and 10% NaCl [wt/vol], and at each pH used for the respective media. All media tested were shown to remain colourless (indicating a total lack of oxygen) following a 7-day period.

5.3.2 Selection of Model Organisms

Halomonas hydrothermalis (DSM-15725) is a Gram-negative, rod-shaped facultative anaerobic strain isolated from low-temperature hydrothermal vent fluid at 2580 m depth in the South Pacific (Kaye et al., 2004). This strain was employed in this study due to its ability to propagate over a wide range of temperatures and salinities, making it a model organism for examining the effect of changing environmental parameters on microbial propagation. *H. hydrothermalis* exhibits cellular division at NaCl concentrations between 0.5% and 22% [wt/vol] (optimal range of 4% to 7% [wt/vol]), pH between 5 and 12 (optimal range of 7 to 8)

and temperatures between 2°C and 40°C (optimal growth at 30°C) (Kaye et al., 2004).

Escherichia coli (DSM-1103) was investigated in this study due to its wide range of growth parameters both aerobically and anaerobically, making it one of the favoured bacterial species when studying energetics (Unden and Bongaerts, 1997).

Carnobacterium pleistocenium (DSM-17715) is a Gram-positive, rod-shaped facultitave anaerobic strain isolated from Pleistocene ice in a permafrost tunnel in Fox, Alaska (Pikuta et al., 2005). This strain was chosen for this study due to its more limited growth parameters relative to *H. hydrothermalis* and *E. coli*. Having a lower growth range of temperature 0 to 28°C (optimal 24°C), and a narrower salinity range 0 to 5% NaCl [wt/vol] (optimal 0.5% [wt/vol]) and a pH range 6.5 to 9.5 (optimal range 7.3 to 7.5) allows for an increased understanding of the effect of multiple extremes over of more restricted parameter space (Pikuta et al., 2005).

5.3.3 Microbial Preparation

Halomonas hydrothermalis

Halomonas hydrothermalis DSM-15725 was secured from the German collection of Microorganisms (DSMZ, Braunschweig, Germany). *H. hydrothermalis* starter cultures were obtained by culturing of the bacterium in minimal marine media (MMM) (Östling et al., 1991), altering the level of glucose to produce a MMM with 0.5% glucose composition, with 1.63% NaCl [wt/vol] (pH 8). Agar plates were prepared using MMM with the addition of Agar bacteriological No. 1 at a concentration of 1.5% [wt/vol] from freezer stock cultures (Section 3.2.1). *H. hydrothermalis* starter cultures were prepared by transferring cells from agar plates to ∼30 mL MMM broth in a 250 mL conical flask with a foam bung. Cultures were grown for 24 hours in a shaking incubator (30°C, 120 rpm).

Anaerobic starter cultures of *H. hydrothermalis* were initiated by culturing on agar plates prepared using MMM with the addition of Agar bacteriological No. 1 at a concentration of 1.5% wt/vol from freezer stock cultures (Section 3.2.1). Starter cultures were prepared by transferring cells from agar plates into anaerobically prepared MMM (see 3.3.2) in serum bottles sealed with

a butyl rubber bung and hand clamped with aluminium caps. Cultures were grown for 24 hours in a shaking incubator (30°C, 120 rpm).

Escherichia coli

E. coli starter cultures were obtained by culturing of the bacterium in Luria-Bertani (LB) medium (DSMZ medium no. 321). Agar plates were prepared using LB with the addition of Agar bacteriological No. 1 at a concentration of 1.5% wt/vol from freezer stock cultures (Section 3.3.2). *E. coli* starter cultures were prepared by transferring cells from agar plates to ∼30 mL LB broth in a 250 mL conical flask with a foil covered foam bung. Cultures were grown for 24 hours in a shaking incubator (30°C, 120 rpm).

Anaerobic starter cultures of *E. coli* were initiated by culturing on agar plates prepared with LB and the addition of Agar bacteriological at a concentration of 1.5% [wt/vol] from freezer stock cultures (Section 3.2.1). Starter cultures were prepared by transferring cells from agar plates into anaerobically prepared LB (see 5.3.2) in serum bottles sealed with a butyl rubber bung and hand clamped with aluminium caps. Cultures were grown for 24 hours in a shaking incubator (30°C, 120 rpm).

Carnobacterium pleistocenium

C. pleistocenium starter cultures were obtained by culturing of the bacterium in modified PYG medium (DSMZ medium no. 104) without the addition of cysteine or resazurin, and without being cooled under CO2 (see 3.3.2). Growth of *C. pleistocenium* on prepared agar plates was limited, therefore starter cultures were initiated by direct inoculation from freezer stock solutions (Section 3.2.1) into prepared modified PYG media. Cultures were grown for 96-hours incubated at 24°C without shaking.

Anaerobic starter cultures of *C. pleistocenium* were initiated by aliquoting 20 μL of aerobically cultures *C. pleistocenium* into ∼30 mL anaerobically prepared PYG media. Cultures were then incubated 24°C without shaking for 96 hours. Following initial incubation, a further 20 μL was transferred into ∼30 mL anaerobically prepared PYG media and incubated for a further 96-hours at 24°C without shaking. This method was employed in order to reduce any oxygen

transferred through aliquoting of initial starter culture from aerobic media.

5.3.4 Growth Assays

Halomonas hydrothermalis

H. hydrothermalis growth assays were started by adding 2 μL of *H. hydrothermalis* starter culture to fresh MMM broth at a range of salinities ($n = 19$) to a total volume of 200 μl in 96-well microplates with the accompanying lid (Greiner Bio-One, Frickenhausen, Germany) for aerobic cultures or with non-gas permeable adhesive PCR plate seals (Thermo-Fisher Scientific) for anaerobic cultures. Nineteen variations of MMM were used with 0.93%, 1.39%, 1.85%, 2.31%, 2.77%, 3.24%, 3.70%, 4.16%, 4.62%, 5.08%, 5.55%, 6.01%, 6.47%, 6.93%, 7.39%, 7.86%, 8.32%, 8.78% and 9.24% NaCl [wt/vol]. Concentrations were established by mixing of MMM with 0% and 10% NaCl [wt/vol] within a 96-well plate. Three variations of culture media based on MMM under salinity ranges were prepared at pH values of 8, 7 and 6 using 1 M hydrochloric acid to lower pH. The pH was measured using a Jenway 3510 pH meter (further information on pH measurements can be found in section 3.3.4).

All *H. hydrothermalis* cultures were incubated at 25, 30, 35, 40 and 45°C to cover a broad range of temperatures from sub-optimal (25°C), through optimal (30°C) to supra-optimal (35, 40 and 45°C) values for *H. hydrothermalis* under otherwise optimal conditions. Cell density was measured over a 24-hour period by OD_{600} measurement using a SPECTROstar Nano Microplate reader (BMG Labtech) and shaken continuously at 1080 rpm. Each culture condition was observed in triplicate within a micro-plate per temperature and pH combination. Medium only controls were included ($n = 5$) for each plate. As a consequence of very low maximal OD_{600} values attained under multiple extremes, it is necessary establish a cut off point for assumed propagation. This study assumes a max OD_{600} value >0.01 to be a result of microbial growth, any value obtained lower than this was deemed to be unreliable.

Escherichia coli

E. coli growth assays were started by adding 2 μL of *E. coli* starter culture to fresh LB broth at a range of salinities ($n = 19$) to a total volume of 200 μL in

96-well microplates with the accompanying lid (Greiner Bio-One, Frickenhausen, Germany) for aerobic cultures or with non-gas permeable adhesive PCR plate seals (Thermo-Fisher Scientific) for anaerobic cultures. Nineteen variations of LB were used with 1%, 2.25%, 2.7%, 3.15%, 3.6%, 4.05%, 4.5%, 4.95%, 5.4%, 5.85%, 6.3%, 6.75%, 7.2%, 7.65%, 8.1%, 8.55%, 9%, 9.45% and 9.9% NaCl [wt/vol]. Concentrations were established by mixing of LB with 0% and 10% NaCl [wt/vol] within a 96-well plate. Four variations of culture media based on LB under salinity ranges were prepared at pH 7, 6, 5 and 4 using 1 M hydrochloric acid to lower pH. The pH was measured using a Jenway 3510 pH meter.

All *E. coli* cultures were incubated at 30, 35, 40 and 45°C to cover a broad range of temperatures from sub-optimal (30 and 35°C) to supra-optimal (40 and 45°C). Optimal temperature for *E. coli* is 37°C. Cell density was measured over a 24-hour period by OD_{600} measurement using a SPECTROstar Nano Microplate reader (BMG Labtech) and shaken continuously at 1080 rpm. Each culture condition was observed in triplicate within a micro-plate per temperature and pH combination. Medium only controls were included ($n = 5$) for each plate. As a consequence of very low maximal OD_{600} values attained under multiple extremes, it is necessary establish a cut of point for assumed propagation. This study assumes a max OD_{600} value >0.01 to be a result of microbial growth, any value obtained lower than this was deemed to be unreliable.

Carnobacterium pleistocenium

C. pleistocenium growth assays were started by adding 2 μl of *C. pleistocenium* starter culture to fresh PYG media at a range of salinities ($n = 11$) to a total volume of 200 μl in 96-well microplates with the accompanying lid (Greiner Bio-One, Frickenhausen, Germany) for aerobic cultures or with non-gas permeable adhesive PCR plate seals (Thermo-Fisher Scientific) for anaerobic cultures. Eleven variations of PYG were used with 0%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% and 5% NaCl [wt/vol]. Concentrations were established by mixing of PYG media with 0% and 10% NaCl [wt/vol] within a 96-well plate. Four variations of culture media based on PYG under salinity ranges were prepared at pH 7.5, 7, 6.5 and 6 using 1 M hydrochloric acid to lower pH. The pH was measured using a Jenway 3510 pH meter.

All *C. pleistocenium* cultures were incubated at 24, 26, 28 and 30°C to cover a range of temperatures from optimal (24°C) to supra-optimal (26, 28 and

30°C). Cell density was measured using a Synergy 2 microplate reader (BioTek Instruments Inc., Vermont, USA) shaken continuously at 1080 rpm. Each culture condition was observed in triplicate within a micro-plate per temperature and pH combination. Medium only controls were included ($n = 5$) for each plate. As a consequence of very low maximal OD_{600} values attained under multiple extremes, it is necessary establish a cut of point for assumed propagation. This study assumes a max OD_{600} value >0.01 to be a result of microbial growth, any value obtained lower than this was deemed to be unreliable.

5.3.5 Data Analysis

The linear relationship between NaCl and mean OD_{600} values were assessed for both aerobic and anaerobic conditions using Pearsons Product-Moment Correlation. Significant differences in measured max OD_{600} values were assessed using a two-sample independent Students t-test. To assess the equality of variance between samples a Bartletts test was performed, for any samples displaying unequal variance a Welchs t-test was performed (marked with an asterisk in the results tables). The Benjamini-Hochberg Procedure was carried out on resulting p-values to decrease the false recovery rate. Statistical analysis and images were performed and created using RStudio v1.1.453 (RStudioTeam, 2018) with the packages 'car', 'ggplot2' and 'gridExtra'.

5.4 Results

5.4.1 Aerobic vs Anaerobic Conditions for Halomonas hydrothermalis Under the Combined Stress of Salinity, pH and Temperature.

To measure the relationship between concentrations of NaCl [wt/vol], variations in pH and temperature on microbial propagation employing different modes of metabolism, *Halomonas hydrothermalis* was cultured under a range of salinities, pH and temperatures under aerobic conditions and anaerobic conditions.

25°C

When cultured under aerobic conditions, *H. hydrothermalis* displayed higher aerobic growth at 25°C than anaerobic growth for all pH variations tested excluding five instances in which anaerobic cultures displayed higher growth (pH 7 at 7.39, 7.86, 8.78 and 9.24% NaCl [wt/vol] and pH 6 at 0.93% NaCl [wt/vol]) (Table 5.1). Only pH 6 and 0.93% NaCl [wt/vol] were significantly different between aerobic and anaerobic conditions (Two-sample independent Students *t*-test) (Table 5.6). *T*-tests revealed significant differences in mean max OD_{600} values between aerobic and anaerobic conditions at salinities 0.93, 1.39, 1.85, 2.31, 2.77, 3.24, 3.7, 4.16, 4.62, 5.08, 5.55, 6.01, 6.47 and 8.78% NaCl [wt/vol] for pH 8. At pH 7, *t*-tests show significant differences between salinities 0.93, 1.39, 1.85, 2.31, 2.77, 3.24, 3.7, 4.16, 4.62, 5.55 and 6.01% NaCl [wt/vol], and for pH 6 at 0.93, 1.39, 1.85, 2.31 and 3.7% NaCl [wt/vol] (Table 5.6). Mean max OD_{600} values obtained under pH 8 conditions at 25°C displayed highly significant strong negative linear correlation with salinity under aerobic growth conditions (r = -0.982, $p < 0.001$; Pearsons product-moment correlation coefficient), and non-significantly weak negative linear correlation under anaerobic conditions (r = -0.351, $p = 0.14$; Pearsons product-moment correlation coefficient) (Figure. 5.1). Mean max OD_{600} values obtained under pH 7 conditions at 25°C displayed highly significant strong negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.914, $p < 0.001$ and r = -0.678, $p <$ 0.01 respectively; Pearsons product-moment correlation coefficient) (Figure 5.2). Mean max OD_{600} values obtained under pH 6 conditions at 25°C displayed highly significant strong negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.61, $p < 0.01$ and r = -0.879, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient) (Figure 5.3).

30°C and 35°C

At 30°C and 35°C, aerobic growth of *H. hydrothermalis* was higher than anaerobic growth at pH 8 and 7 in all instances excluding one (35°C, pH7 and 2.31% NaCl [wt/vol]) which was not statistically significantly different (Two-sample independent Students *t*-test) (Table 5.8). Anaerobic growth at 30°C and 35°C under pH 6 conditions were higher than aerobic conditions in all instances in which growth occurred excluding one (30°C and 2.77% NaCl [wt/vol]), which

was significantly different (Welchs t-test) (Table 5.7).

30°C

At 30°C, t-tests revealed significant differences in mean max OD_{600} values between aerobic and anaerobic conditions at all salinities excluding one (8.32% NaCl [wt/vol]) under pH 8 conditions, and all salinities excluding four (1.39, 2.31, 3.24 and 9.24% NaCl [wt/vol]) for pH 7 conditions (Table 5.7). Significant differences under pH 6 conditions were at 0.93, 2.31 and 4.16% NaCl [wt/vol] (t-test) (Table 5.7). At pH 8 and 30°C, mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under aerobic growth conditions (r = -0.961, $p < 0.001$; Pearsons product-moment correlation coefficient), and were not significantly correlated under anaerobic conditions (r = -0.046, p = 0.851; Pearsons product-moment correlation coefficient) (Figure 5.1). At pH 7 and 30°C, mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under aerobic conditions (r = -0.908, $p < 0.001$; Pearsons product-moment correlation coefficient), and significant moderate negative linear correlation under anaerobic conditions (r = -0.569, $p < 0.05$; Pearsons product-moment correlation coefficient) (Figure 5.2). At pH 6 and 30°C, mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.634, $p < 0.01$ and r = -0.638, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient) (Figure 5.3).

35°C

At 35°C, aerobic growth was higher than anaerobic growth in all instances with significant differences in mean max OD_{600} values between aerobic and anaerobic conditions at all salinities excluding three (5.55, 6.01 and 8.32% NaCl [wt/vol]) under pH 8 conditions (t-test) (Table 5.8). T-tests revealed significant differences in mean OD_{600} values between salinities 0.93, 1.39, 2.77, 4.16, 4.62, 5.08, 5.55, 6.47, 6.93 and 7.39% NaCl [wt/vol] under pH 7 conditions, and at no significant differences were measured at pH 6 (Table 5.8). At pH 8 and 35°C, mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under aerobic growth conditions (r = -0.927, $p < 0.001$; Pearsons product-moment correlation coefficient), and significantly moderate negative linear correlation under anaerobic conditions (r = -0.575, $p < 0.05$; Pearsons product-moment correlation coefficient) (Figure 5.1). At pH 7 and 35°C, mean max OD_{600} values displayed significantly moderate negative

linear correlation with salinity under aerobic conditions (r = -0.501, $p < 0.05$; Pearsons product-moment correlation coefficient), and anaerobic conditions were not shown to be significantly correlated (Pearsons product-moment correlation coefficient) (Figure 5.2). At pH 6 and 35°C, mean max OD_{600} values displayed non-significant moderate negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.349, $p = 0.143$ and r = -0.46, $p = 0.055$ respectively; Pearsons product-moment correlation coefficient) (Figure 5.3).

40°C

At 40°C, differences in growth at pH 8 vary without pattern between aerobic and anaerobic conditions (Table 5.4), and t-tests revealed no instances of significant differences (Table 5.9). Anaerobic conditions yielded growth under pH 7 in more instances than aerobic growth, particularly within the lower optimal salinity range for *H. hydrothermalis* (4% to 7% [wt/vol]) (Table 5.4), and two-sample independent Students t-test revealed no significant differences between instances of aerobic and anaerobic growth (Table 5.9). Under pH 6 conditions, aerobic conditions yielded growth in more instances than anaerobic conditions particularly within the optimal salinity range for *H. hydrothermalis* (Table 5.4), and two-sample independent students t-test revealed no significant differences between instances of aerobic and anaerobic growth (Table 5.9). At pH 8 and 40°C, mean max OD_{600} values were not shown to be significantly correlated under either aerobic or anaerobic conditions (r = -0.199, $p = 0.412$ and r = -0.192, p = 0.43 respectively; Pearsons product-moment correlation coefficient) (Figure 5.1). At pH 7 and 40°C, mean max OD_{600} values displayed non-significantly weak negative linear correlation with salinity under anaerobic conditions (r = -0.328, $p = 0.17$; Pearsons product-moment correlation coefficient) (Figure 5.2). At pH 6 and 40°C, mean max OD_{600} values displayed non-significant moderate negative linear correlation with salinity under aerobic conditions (r = -0.417, $p = 0.075$; Pearsons product-moment correlation coefficient) (Figure 5.3).

45°C

As with data collected at 40°C, growth values obtained at 45°C display a similar pattern for pH 7 and 6 where anaerobic growth is measured under pH 7 particularly within the lower optimal range for *H. hydrothermalis* (4% to 7%

[wt/vol]), and more instances of aerobic growth at pH 6 (Table 5.5). Two-sample independent Students t-tests revealed no significant differences between instances of aerobic and anaerobic growth (Table 5.10). Under pH 8 conditions, growth was not determinable for aerobic and anaerobic cultures excluding one instance (anaerobic and 2.77% NaCl [wt/vol]).

Table (5.1) *Mean and Standard Deviation ± (n = 3) of the mean max OD_{600} values for* H. hydrothermalis *cultured at 25°C under a range of pH values and salinity (% NaCl [wt/vol]) (n = 19).*

25°C						
	pH 8	**pH 8**	**pH 7**	**pH 7**	**pH 6**	**pH 6**
NaCl (%)	+	-	+	-	+	-
0.93	**2.140 ± 0.19**	0.081 ± 0.001	**1.112 ± 0.08**	0.149 ± 0.14	0.022 ± 0.01	**0.040 ± 0.01**
1.39	**2.071 ± 0.08**	0.070 ± 0.004	**1.725 ± 0.03**	0.080 ± 0.01	**0.068 ± 0.02**	0.025 ± 0.003
1.85	**2.040 ± 0.18**	0.075 ± 0.01	**1.764 ± 0.20**	0.082 ± 0.02	**0.089 ± 0.01**	0.029 ± 0.02
2.31	**1.743 ± 0.04**	0.058 ± 0.003	**1.482 ± 0.19**	0.068 ± 0.01	**0.077 ± 0.02**	0.028 ± 0.01
2.77	**1.840 ± 0.17**	0.059 ± 0.001	**1.720 ± 0.03**	0.078 ± 0.02	**0.047 ± 0.001**	0.028 ± 0.02
3.24	**1.843 ± 0.19**	0.059 ± 0.002	**1.560 ± 0.03**	0.213 ± 0.23	**0.029 ± 0.005**	0.023 ± 0.01
3.7	**1.739 ± 0.21**	0.398 ± 0.59	**1.329 ± 0.07**	0.079 ± 0.01	**0.023 ± 0.003**	0.012 ± 0.003
4.16	**1.515 ± 0.07**	0.050 ± 0.01	**0.883 ± 0.16**	0.044 ± 0.01	**0.024 ± 0.01**	0.012 ± 0.01
4.62	**1.461 ± 0.29**	0.051 ± 0.004	**0.995 ± 0.18**	0.054 ± 0.01	**0.016 ± 0.003**	-
5.08	**1.295 ± 0.11**	0.061 ± 0.01	**0.607 ± 0.30**	0.061 ± 0.002	**0.013 ± 0.002**	-
5.55	**1.139 ± 0.21**	0.113 ± 0.11	**0.324 ± 0.08**	0.052 ± 0.01	-	-
6.01	**0.835 ± 0.13**	0.051 ± 0.01	**0.124 ± 0.01**	0.043 ± 0.01	-	-
6.47	**0.720 ± 0.19**	0.041 ± 0.001	**0.069 ± 0.01**	0.047 ± 0.01	**0.012 ± 0.01**	-
6.93	**0.516 ± 0.22**	0.032 ± 0.003	**0.039 ± 0.01**	0.028 ± 0.01	**0.010 ± 0.001**	-
7.39	**0.289 ± 0.14**	0.026 ± 0.004	0.027 ± 0.002	**0.032 ± 0.01**	-	-
7.86	**0.110 ± 0.05**	0.026 ± 0.004	0.018 ± 0.004	**0.028 ± 0.005**	**0.011 ± 0.01**	-
8.32	**0.067 ± 0.04**	0.016 ± 0.01	**0.025 ± 0.002**	0.016 ± 0.01	**0.017 ± 0.01**	-
8.78	**0.025 ± 0.001**	0.015 ± 0.001	0.016 ± 0.004	**0.038 ± 0.02**	**0.023 ± 0.02**	-
9.24	**0.021 ± 0.01**	-	0.012 ± 0.002	**0.023 ± 0.01**	**0.033 ± 0.03**	-

Table (5.2) *Mean and Standard Deviation ± (n = 3) of the mean max OD_{600} values for* H. hydrothermalis *cultured at 30°C under a range of pH values and salinity (% NaCl [wt/vol]) (n = 19).*

30°C						
	pH 8	**pH 8**	**pH 7**	**pH 7**	**pH 6**	**pH 6**
NaCl (%)	+	-	+	-	+	-
0.93	**3.047 ± 0.16**	0.113 ± 0.01	**2.142 ± 0.05**	0.121 ± 0.01	0.017 ± 0.002	**0.031 ± 0.0005**
1.39	**2.992 ± 0.39**	0.101 ± 0.001	**2.152 ± 0.02**	0.892 ± 1.26	0.022 ± 0.002	**0.211 ± 0.30**
1.85	**2.733 ± 0.57**	0.097 ± 0.01	**2.152 ± 0.04**	0.159 ± 0.09	0.083 ± 0.02	**0.276 ± 0.23**
2.31	**2.888 ± 0.51**	0.094 ± 0.01	**2.138 ± 0.03**	0.601 ± 0.78	0.089 ± 0.02	**0.264 ± 0.01**
2.77	**2.628 ± 0.21**	0.092 ± 0.01	**2.061 ± 0.08**	0.089 ± 0.01	**0.060 ± 0.01**	0.036 ± 0.004
3.24	**2.727 ± 0.44**	0.098 ± 0.01	**2.003 ± 0.05**	0.715 ± 1.04	0.046 ± 0.01	**0.372 ± 0.28**
3.7	**2.630 ± 0.38**	0.086 ± 0.01	**1.999 ± 0.03**	0.220 ± 0.23	0.028 ± 0.002	**0.282 ± 0.12**
4.16	**2.544 ± 0.44**	0.097 ± 0.03	**1.993 ± 0.03**	0.252 ± 0.17	0.018 ± 0.003	**0.135 ± 0.01**
4.62	**2.151 ± 0.29**	0.114 ± 0.02	**1.907 ± 0.05**	0.088 ± 0.01	0.016 ± 0.01	**0.028 ± 0.003**
5.08	**2.117 ± 0.20**	0.114 ± 0.01	**1.806 ± 0.03**	0.134 ± 0.02	0.010 ± 0.003	**0.016 ± 0.01**
5.55	**2.051 ± 0.25**	0.089 ± 0.01	**1.738 ± 0.08**	0.092 ± 0.02	-	**0.011 ± 0.01**
6.01	**1.860 ± 0.14**	0.101 ± 0.03	**1.730 ± 0.01**	0.263 ± 0.15	-	-
6.47	**1.491 ± 0.36**	0.137 ± 0.03	**1.379 ± 0.16**	0.131 ± 0.06	**0.012 ± 0.01**	-
6.93	**1.537 ± 0.31**	0.164 ± 0.10	**1.300 ± 0.05**	0.089 ± 0.01	-	-
7.39	**1.182 ± 0.41**	0.174 ± 0.14	**1.037 ± 0.04**	0.138 ± 0.07	**0.013 ± 0.02**	-
7.86	**0.668 ± 0.13**	0.123 ± 0.03	**0.416 ± 0.09**	0.068 ± 0.004	-	-
8.32	**0.332 ± 0.20**	0.112 ± 0.02	**0.120 ± 0.01**	0.041 ± 0.004	**0.012 ± 0.001**	-
8.78	**0.101 ± 0.02**	0.059 ± 0.01	**0.082 ± 0.01**	0.034 ± 0.02	-	-
9.24	**0.075 ± 0.01**	0.046 ± 0.01	**0.072 ± 0.01**	0.050 ± 0.01	-	-

Table (5.3) *Mean and Standard Deviation ± (n = 3) of the mean max OD_{600} values for* H. hydrothermalis *cultured at 35°C under a range of pH values and salinity (% NaCl [wt/vol]) (n = 19).*

35°C						
	pH 8	**pH 8**	**pH 7**	**pH 7**	**pH 6**	**pH 6**
NaCl (%)	+	-	+	-	+	-
0.93	**2.340 ± 0.51**	0.597 ± 0.37	**2.039 ± 0.23**	0.078 ± 0.03	-	**0.050 ± 0.01**
1.39	**2.822 ± 0.41**	0.587 ± 0.45	**1.260 ± 0.25**	0.256 ± 0.04	-	**0.056 ± 0.03**
1.85	**3.362 ± 0.11**	0.708 ± 0.23	**0.803 ± 0.17**	0.575 ± 0.64	0.013 ± 0.002	**0.356 ± 0.56**
2.31	**3.258 ± 0.26**	0.724 ± 0.43	0.414 ± 0.04	**0.439 ± 0.08**	0.026 ± 0.02	**0.042 ± 0.01**
2.77	**2.733 ± 0.43**	0.229 ± 0.12	**0.971 ± 0.25**	0.302 ± 0.07	0.016 ± 0.01	**0.682 ± 0.15**
3.24	**2.842 ± 0.44**	0.328 ± 0.23	**1.025 ± 0.34**	0.484 ± 0.26	0.012 ± 0.002	**0.679 ± 0.14**
3.7	**2.780 ± 0.49**	0.173 ± 0.01	**1.076 ± 0.31**	0.323 ± 0.02	0.012 ± 0.002	**0.271 ± 0.15**
4.16	**2.879 ± 0.28**	0.143 ± 0.03	**1.170 ± 0.27**	0.445 ± 0.10	0.012 ± 0.002	**0.016 ± 0.003**
4.62	**2.130 ± 0.12**	0.225 ± 0.04	**1.293 ± 0.43**	0.278 ± 0.07	0.011 ± 0.005	**0.013 ± 0.003**
5.08	**2.008 ± 0.22**	0.239 ± 0.08	**1.469 ± 0.12**	0.356 ± 0.05	-	**0.012 ± 0.01**
5.55	**1.593 ± 0.23**	0.745 ± 0.79	**1.417 ± 0.12**	0.241 ± 0.05	-	-
6.01	**1.407 ± 0.14**	0.698 ± 0.62	**1.340 ± 0.05**	0.884 ± 1.0	-	-
6.47	**1.311 ± 0.04**	0.238 ± 0.07	**1.286 ± 0.04**	0.167 ± 0.13	**0.013 ± 0.01**	-
6.93	**1.167 ± 0.05**	0.255 ± 0.01	**1.267 ± 0.03**	0.343 ± 0.04	-	-
7.39	**1.159 ± 0.08**	0.265 ± 0.02	**1.290 ± 0.04**	0.365 ± 0.18	-	-
7.86	**1.144 ± 0.06**	0.266 ± 0.05	**0.676 ± 0.35**	0.157 ± 0.02	-	-
8.32	**0.803 ± 0.62**	0.159 ± 0.03	**0.092 ± 0.04**	0.078 ± 0.05	-	-
8.78	**0.171 ± 0.03**	0.090 ± 0.003	**0.088 ± 0.01**	0.062 ± 0.001	-	-
9.24	**0.148 ± 0.01**	0.046 ± 0.01	**0.080 ± 0.02**	0.065 ± 0.02	**0.012 ± 0.002**	-

Table (5.4) *Mean and Standard Deviation ± (n = 3) of the mean max OD_{600} values for* H. hydrothermalis *cultured at 40°C under a range of pH values and salinity (% NaCl [wt/vol]) (n = 19).*

40°C						
	pH 8	**pH 8**	**pH 7**	**pH 7**	**pH 6**	**pH 6**
NaCl (%)	+	-	+	-	+	-
0.93	**0.010 ± 0.01**	-	-	-	-	**0.010 ± 0.003**
1.39	0.019 ± 0.004	**0.019 ± 0.01**	-	**0.017 ± 0.005**	-	-
1.85	0.021 ± 0.01	**0.021 ± 0.01**	**0.019 ± 0.01**	0.017 ± 0.01	-	-
2.31	**0.028 ± 0.03**	0.011 ± 0.001	0.015 ± 0.01	**0.017 ± 0.01**	-	-
2.77	**0.820 ± 1.40**	0.018 ± 0.004	-	-	-	-
3.24	-	-	-	**0.028 ± 0.03**	-	-
3.7	**0.020 ± 0.01**	0.013 ± 0.004	-	**0.011 ± 0.001**	-	-
4.16	**0.480 ± 0.55**	0.025 ± 0.01	-	**0.012 ± 0.01**	**0.011 ± 0.004**	-
4.62	**0.141 ± 0.10**	0.032 ± 0.001	-	**0.021 ± 0.01**	**0.014 ± 0.004**	-
5.08	0.041 ± 0.04	**0.062 ± 0.01**	-	**0.014 ± 0.004**	**0.011 ± 0.001**	-
5.55	**0.166 ± 0.16**	0.066 ± 0.01	-	-	**0.012 ± 0.001**	-
6.01	**0.323 ± 0.05**	0.178 ± 0.09	-	**0.016 ± 0.01**	**0.013 ± 0.001**	-
6.47	**0.136 ± 0.09**	0.084 ± 0.01	**0.010 ± 0.01**	-	**0.011 ± 0.002**	0.011 ± 0.001
6.93	0.036 ± 0.04	**0.045 ± 0.02**	-	**0.011 ± 0.01**	-	-
7.39	-	**0.042 ± 0.02**	-	-	-	-
7.86	-	**0.029 ± 0.01**	-	-	-	-
8.32	0.014 ± 0.02	0.014 ± 0.003	0.012 ± 0.002	**0.019 ± 0.01**	**0.017 ± 0.004**	-
8.78	-	-	**0.013 ± 0.004**	-	**0.013 ± 0.004**	-
9.24	-	-	-	**0.013 ± 0.01**	-	-

Table (5.5) *Mean and Standard Deviation ± (n = 3) of the mean max OD_{600} values for* H. hydrothermalis *cultured at 45°C under a range of pH values and salinity (% NaCl [wt/vol]) (n = 19).*

45°C						
	pH 8	**pH 8**	**pH 7**	**pH 7**	**pH 6**	**pH 6**
NaCl (%)	+	-	+	-	+	-
0.93	-	-	-	-	-	-
1.39	-	-	-	**0.021 ± 0.03**	-	-
1.85	-	-	-	-	-	-
2.31	-	-	-	**0.011 ± 0.001**	-	-
2.77	-	**0.012 ± 0.01**	-	-	-	**0.011 ± 0.004**
3.24	-	-	-	-	-	-
3.7	-	-	-	-	-	**0.011 ± 0.01**
4.16	-	-	-	**0.011 ± 0.001**	-	-
4.62	-	-	-	**0.015 ± 0.01**	-	**0.016 ± 0.004**
5.08	-	-	-	**0.010 ± 0.01**	**0.010 ± 0.004**	-
5.55	-	-	-	-	**0.012 ± 0.002**	-
6.01	-	-	-	**0.010 ± 0.003**	**0.014 ± 0.01**	-
6.47	-	-	-	**0.016 ± 0.01**	**0.012 ± 0.002**	0.011 ± 0.01
6.93	-	-	-	-	**0.015 ± 0.01**	-
7.39	-	-	-	-	**0.011 ± 0.01**	-
7.86	-	-	-	**0.010 ± 0.005**	-	**0.018 ± 0.02**
8.32	-	-	0.010 ± 0.004	**0.013 ± 0.01**	**0.015 ± 0.001**	0.012 ± 0.01
8.78	-	-	-	**0.011 ± 0.01**	-	-
9.24	-	-	**0.013 ± 0.01**	-	**0.012 ± 0.01**	-

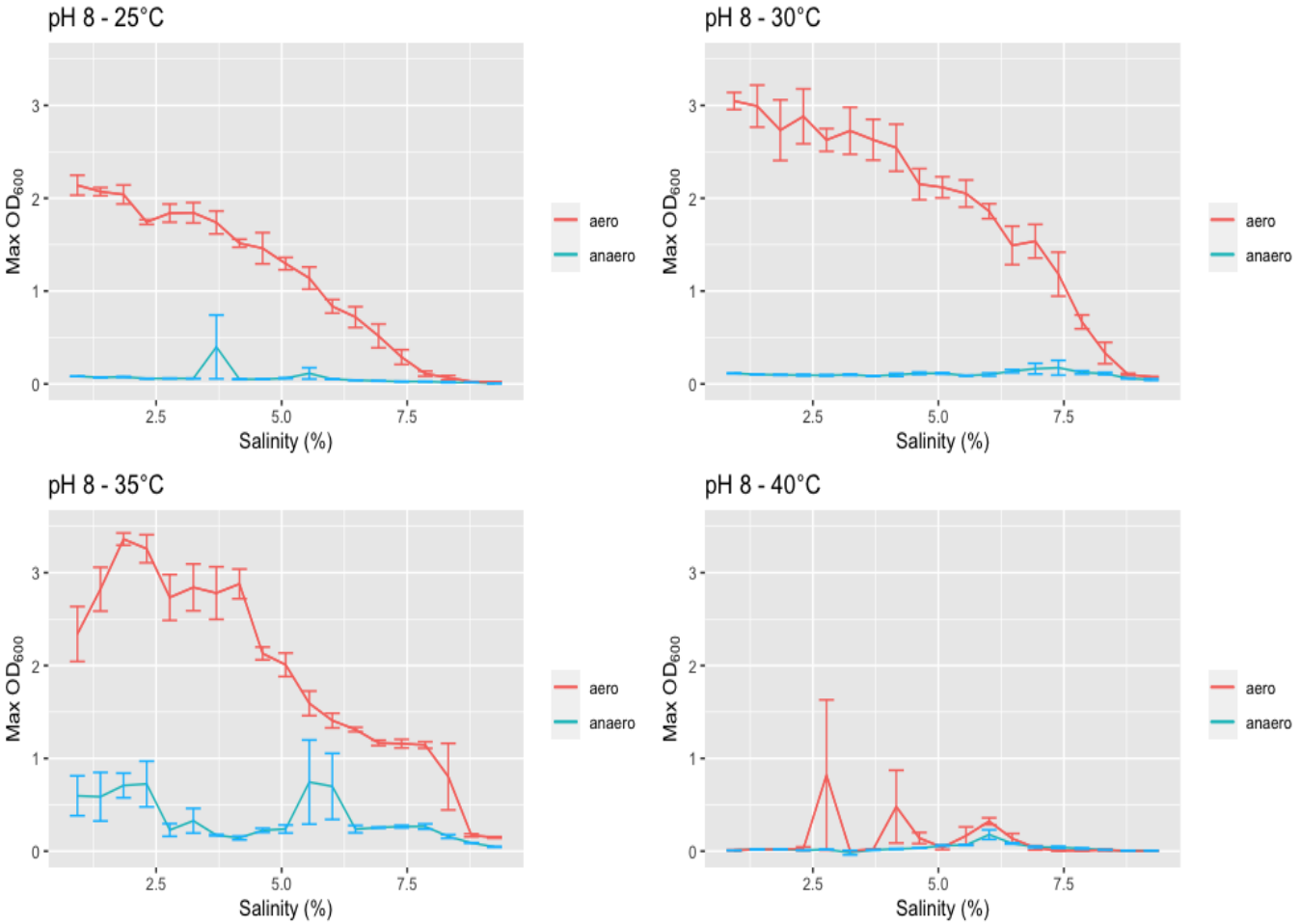

Figure (5.1) *Graph displaying mean maximal OD_{600} values obtained under pH 8 and a range of salinities (% NaCl [wt/vol] (n = 19), and temperatures for* H. hydrothermalis. *Data presented as mean max OD_{600} values ± standard error of the means (SE) (n = 3).*

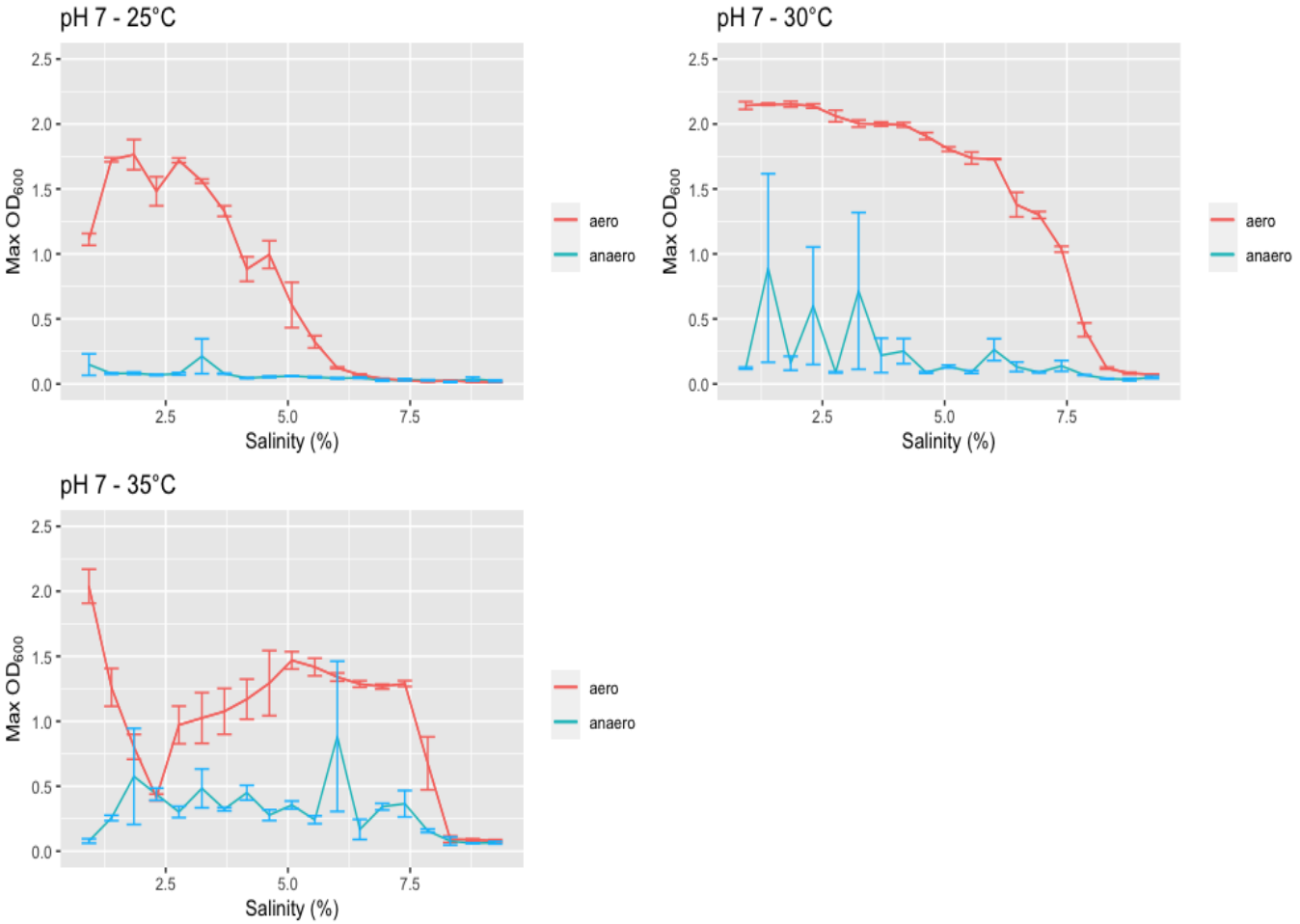

Figure (5.2) *Graph displaying mean maximal OD_{600} values obtained under pH 7 and a range of salinities (% NaCl [wt/vol] (n = 19), and temperatures for* H. hydrothermalis. *Data presented as mean max OD_{600} values ± standard error of the means (SE) (n = 3).*

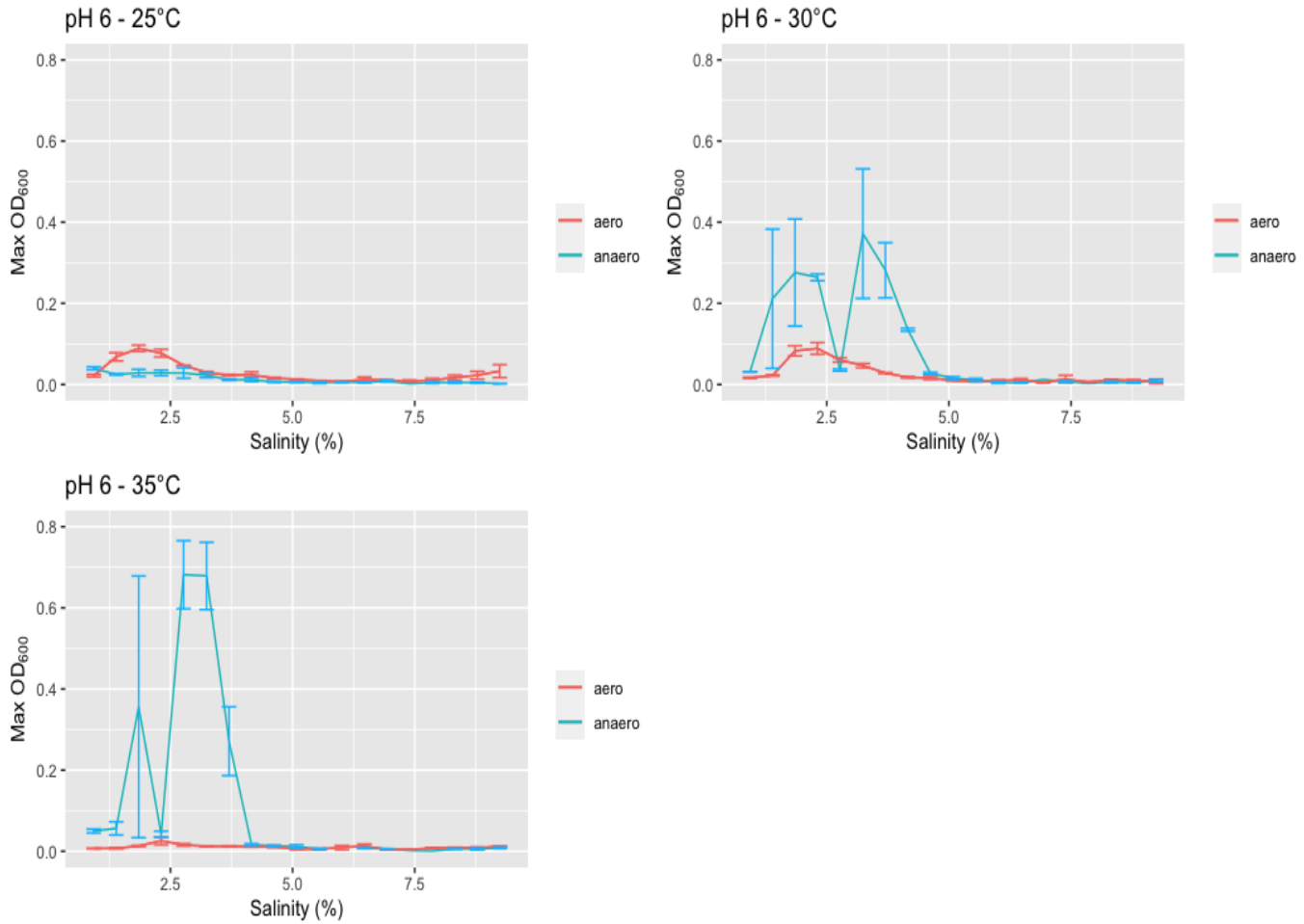

Figure (5.3) *Graph displaying mean maximal OD_{600} values obtained under pH 6 and a range of salinities (% NaCl [wt/vol] (n = 19), and temperatures for* H. hydrothermalis. *Data presented as mean max OD_{600} values ± standard error of the means (SE) (n = 3).*

Table (5.6) *T-test results comparing aerobic and anaerobic maximal OD_{600} values obtained for* H. hydrothermalis *cultured under a range of pH conditions and salinities (% NaCl [wt/vol]) (n = 19) at 25°C.*
Green indicates instances in which neither condition yielded measurable data, red indicates no aerobic growth and blue indicates no anaerobic growth.
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

25°C NaCl (%)	**pH 8**	**pH 7**	**pH 6**
0.93	$t(2) = 19.1, p < 0.01$*	$t(4) = 10.2, p < 0.01$	$t(4) = -3.8, p < 0.05$
1.39	$t(2) = 45.5, p < 0.01$*	$t(4) = 97.8, p < 0.0001$	$t(2) = 4.2, p < 0.05$*
1.85	$t(2) = 19.3, p < 0.01$*	$t(2) = 14.5, p < 0.05$*	$t(4) = 5.2, p < 0.01$
2.31	$t(2) = 65.9, p < 0.01$*	$t(2) = 12.6, p < 0.05$*	$t(4) = 4.2, p < 0.05$
2.77	$t(2) = 18.2, p < 0.01$*	$t(4) = 80.1, p < 0.0001$	$t(2) = 1.5, p = 0.282$*
3.24	$t(2) = 16.3, p < 0.01$*	$t(2) = 10, p < 0.05$*	$t(4) = 1.1, p = 0.339$
3.7	$t(4) = 3.7, p < 0.05$	$t(2) = 30.7, p < 0.01$*	$t(4) = 4.4, p < 0.05$
4.16	$t(2) = 34.1, p < 0.01$*	$t(2) = 8.9, p < 0.05$*	$t(4) = 1.5, p = 0.207$
4.62	$t(2) = 8.4, p < 0.05$*	$t(2) = 8.9, p < 0.05$*	
5.08	$t(2) = 18.5, p < 0.01$*	$t(2) = 3.1, p = 0.119$*	
5.55	$t(4) = 7.6, p < 0.01$	$t(2) = 5.7, p < 0.05$*	
6.01	$t(2) = 10.7, p < 0.05$*	$t(4) = 10.4, p < 0.01$	
6.47	$t(4) = 6.1, p < 0.01$	$t(4) = 2.5, p = 0.104$	
6.93	$t(2) = 3.8, p = 0.075$*	$t(4) = 1.4, p = 0.263$	
7.39	$t(2) = 3.3, p = 0.089$*	$t(4) = -0.7, p = 0.507$	
7.86	$t(2) = 3.1, p = 0.092$*	$t(3) = -2.7, p = 0.104$	
8.32	$t(2) = 2.1, p = 0.165$*	$t(4) = 2, p = 0.147$	
8.78	$t(2) = 10.6, p < 0.01$*	$t(4) = -1.5, p = 0.236$	
9.24		$t(3) = -1.2, p = 0.326$	

Table (5.7) *T-test results comparing aerobic and anaerobic maximal OD_{600} values obtained for* H. hydrothermalis *cultured under a range of pH conditions and salinities (% NaCl [wt/vol]) (n = 19) at 30°C.*
Green indicates instances in which neither condition yielded measurable data, red indicates no aerobic growth and blue indicates no anaerobic growth.
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

30°C **NaCl (%)**	**pH 8**	**pH 7**	**pH 6**
0.93	$t(2) = 31.9, p < 0.01$*	$t(4) = 66.1, p < 0.0001$	$t(4) = 11.1, p < 0.01$*
1.39	$t(2) = 12.8, p < 0.05$*	$t(2) = 1.7, p = 0.224$*	$t(2) = 1.1, p = 0.432$*
1.85	$t(2) = 8.1, p < 0.05$*	$t(4) = 34.3, p < 0.0001$	$t(2) = 1.5, p = 0.360$*
2.31	$t(2) = 9.4, p < 0.05$*	$t(2) = 3.4, p = 0.0855$*	$t(4) = 10.6, p < 0.01$
2.77	$t(2) = 20.7, p < 0.01$*	$t(2) = 44.1, p < 0.001$*	$t(4) = 4.2$, p = 0.061*
3.24	$t(2) = 10.4, p < 0.05$*	$t(2) = 2.1, p = 0.175$*	$t(2) = 2, p = 0.316$*
3.7	$t(2) = 11.6, p < 0.05$*	$t(2) = 13.3, p < 0.01$*	$t(2) = 3.7, p = 0.233$*
4.16	$t(2) = 9.6, p < 0.05$*	$t(4) = 17.7, p < 0.001$	$t(4) = 29.1, p < 0.001$
4.62	$t(2) = 12.1$, p < 0.05*	$t(4) = 67, p < 0.0001$	$t(3) = -2.2, p = 0.298$
5.08	$t(2) = 17.6, p < 0.01$*	$t(4) = 82.8, p < 0.0001$	$t(4) = -1.3, p = 0.360$
5.55	$t(2) = 13.5, p < 0.05$*	$t(4) = 34.9, p < 0.0001$	
6.01	$t(4) = 21.4, p < 0.001$	$t(4) = 17.4, p < 0.001$	
6.47	$t(2) = 6.5, p < 0.05$*	$t(4) = 12.3, p < 0.001$	
6.93	$t(4) = 7.2, p < 0.01$	$t(4) = 45, p < 0.0001$	
7.39	$t(4) = 4, p < 0.05$	$t(4) = 19.1, p < 0.001$	
7.86	$t(4) = 7.1, p < 0.01$	$t(2) = 6.6, p < 0.05$*	
8.32	$t(2) = 1.9, p = 0.195$*	$t(4) = 9.8, p < 0.001$	
8.78	$t(4) = 3.2, p < 0.05$	$t(4) = 4.1, p < 0.05$	
9.24	$t(3) = 3.4, p < 0.05$	$t(3) = 2.9, p = 0.076$	

Table (5.8) *T-test results comparing aerobic and anaerobic maximal OD_{600} values obtained for* H. hydrothermalis *cultured under a range of pH conditions and salinities (% NaCl [wt/vol]) (n = 19) at 35°C.*
Green indicates instances in which neither condition yielded measurable data, red indicates no aerobic growth and blue indicates no anaerobic growth.
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

35°C **NaCl (%)**	**pH 8**	**pH 7**	**pH 6**
0.93	$t(4) = 4.8$, **p <0.05**	$t(2) = 14.9$, **p <0.05***	
1.39	$t(4) = 6.3$, **p <0.01**	$t(2) = 6.8$, **p <0.05***	
1.85	$t(4) = 18$, **p <0.001**	$t(4) = 0.6$, $p = 0.651$	$t(2) = 1.1$, $p = 0.491$*
2.31	$t(4) = 8.8$, **p <0.01**	$t(4) = -0.5$, $p = 0.712$	$t(4) = 1.3$, $p = 0.364$
2.77	$t(4) = 9.8$, **p <0.01**	$t(4) = 4.4$, **p <0.05**	*$t(2) = 8$, $p = 0.061$*
3.24	$t(4) = 8.8$, **p <0.01**	$t(4) = 2.2$, $p = 0.134$	$t(2) = 8$, *$p = 0.061$**
3.7	$t(2) = 9.2$, **p <0.05***	$t(2) = 4.3$, $p = 0.086$*	$t(2) = 3.1$, $p = 0.203$*
4.16	$t(2) = 17$, **p <0.01***	$t(4) = 4.4$, **p <0.05**	$t(3) = -0.2$, $p = 0.254$
4.62	$t(4) = 26.4$, **p <0.001**	$t(4) = 4$, **p <0.05**	$t(4) = -0.5$, $p = 0.653$
5.08	$t(4) = 13.2$, **p <0.001**	$t(4) = 15.1$, **p <0.001**	
5.55	$t(4) = 1.8$, $p = 0.155$	$t(4) = 16$, **p <0.0001**	
6.01	$t(4) = 2$, $p = 0.138$	$t(2) = 0.8$, $p = 0.608$*	
6.47	$t(4) = 23.3$, **p <0.001**	$t(4) = 13.7$, **p <0.001**	
6.93	$t(4) = 31.1$, **p <0.001**	$t(4) = 29.3$, **p <0.001**	
7.39	$t(4) = 18.6$, **p <0.001**	$t(4) = 8.9$, **p <0.01**	
7.86	$t(4) = 19.5$, **p <0.001**	$t(2) = 2.5$, $p = 0.169$*	
8.32	$t(2) = 1.8$, $p = 0.214$*	$t(4) = 0.3$, $p = 0.746$	
8.78	$t(2) = 4.9$, **p <0.05***	$t(2) = 0.09$, $p = 0.116$*	
9.24	$t(4) = 10.7$, **p <0.01**	$t(4) = 1.1$, $p = 0.424$	

Table (5.9) *T-test results comparing aerobic and anaerobic maximal OD_{600} values obtained for* H. hydrothermalis *cultured under a range of pH conditions and salinities (% NaCl [wt/vol]) (n = 19) at 40°C.*
Green indicates instances in which neither condition yielded measurable data, red indicates no aerobic growth and blue indicates no anaerobic growth.
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

40°C NaCl (%)	pH 8	pH 7	pH 6
0.93			
1.39	*t(3) = -0.1*, p = 0.989		
1.85	$t(3) = -0.1, p = 0.937$	$t(4) = 0.5, p = 0.869$	
2.31	$t(2) = 0.9, p = 0.715$*	$t(4) = -0.2, p = 0.867$	
2.77	$t(2) = 1, p = 0.715$*		
3.24			
3.7	$t(3) = 1.6, p = 0.684$		
4.16	$t(1) = 1.2, p = 0.714$*		
4.62	$t(2) = 1.8, p = 0.684$*		
5.08	$t(3) = -0.7, p = 0.527$		
5.55	$t(2) = 1.1, p = 0.685$*		
6.01	$t(3) = 2, p = 0.726$		
6.47	$t(2) = 1, p = 0.715$*		$t(4) = 0.7, p = 0.608$
6.93	$t(4) = -0.4, p = 0.728$		
7.39			
7.86			
8.32	$t(4) = -0.01, p = 0.996$	$t(4) = -1.1, p = 0.561$	
8.78			
9.24			

Table (5.10) *T-test results comparing aerobic and anaerobic maximal OD_{600} values obtained for* H. hydrothermalis *cultured under a range of pH conditions and salinities (% NaCl [wt/vol]) (n = 19) at 45°C.*
Green indicates instances in which neither condition yielded measurable data, red indicates no aerobic growth and blue indicates no anaerobic growth.
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

45°C NaCl (%)	pH 8	pH 7	pH 6
0.93			
1.39			
1.85			
2.31			
2.77			
3.24			
3.7			
4.16			
4.62			
5.08			
5.55			
6.01			
6.47			$t(4) = 0.3, p = 0.863$
6.93			
7.39			
7.86			
8.32		$t(4) = -0.7, p = 0.665$	$t(2) = 1, p = 0.719$
8.78			
9.24			

5.4.2 Aerobic vs Anaerobic Conditions for Escherichia coli Under the Combined Stress of Salinity, pH and Supra-optimal Temperatures.

To measure the relationship between concentrations of NaCl [wt/vol], variations in pH and temperature on microbial propagation employing different modes of metabolism *Escherichia coli* was cultured under a range of salinities, pH and temperatures under aerobic conditions and anaerobic conditions.

30°C

When cultured aerobically, *E. coli* displayed higher growth at 30°C at the lower salinity range at pH 7, 6 and 5 than under anaerobic conditions.

pH 7: Higher mean max OD_{600} values were observed under aerobic conditions than for anaerobic conditions for salinities 1, 2.25, 2.7, 3.15, 3.6, 4.05, 4.5, 4.95, 5.4, 5.85, 6.3 and 6.75% NaCl [wt/vol] at pH 7 (Table 5.11). *T*-tests revealed significant differences in mean max OD_{600} values between aerobic and anaerobic conditions at salinities 1 and 4.5% NaCl [wt/vol] at pH 7 (Table 5.15). Higher mean max OD_{600} values were observed under anaerobic conditions for higher salinities at pH 7 (7.2, 9, 9.45 and 9.9% NaCl [wt/vol], excluding instances of which there was no growth for either condition (8.1 and 8.55%) (Table 5.11). However, two-sample independent Students *t*-test revealed no significant differences in mean max OD_{600} values between aerobic and anaerobic cultures at these salinities (Table 5.15). Mean max OD_{600} values obtained under pH 7 conditions at 30°C displayed highly significant strong negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.945, $p < 0.001$ and r = -0.93, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient) (Figure 5.4).

pH 6: At pH 6, higher mean max OD_{600} values were observed for aerobic cultures at lower salinities (1, 2.25, 2.7, 3.15, 3.6, 4.05, 4.5, 4.95, 5.4, 5.85 and 6.3% NaCl [wt/vol] (Table 5.11), with *t*-tests revealing significant differences in mean max OD_{600} values at 1, 2.25, 4.05, 4.5, 4.96, 5.4 and 5.85% NaCl [wt/vol] (Table 5.15). Higher mean max OD_{600} values were observed under anaerobic conditions at pH 6 at higher salinities (6.75, 7.2, 7.65, 8.1, 8.55, 9 and 9.45% NaCl [wt/vol]).

However, t-tests revealed that these differences were not significant (Table 5.15). Mean max OD_{600} values obtained under pH 6 conditions at 30°C displayed highly significant strong negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.872, $p < 0.001$ and r = -0.94, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient) (Figure 5.5).

pH 5: Under pH 5 conditions, higher mean max OD_{600} values were observed under anaerobic conditions for higher salinities (4.5, 4.95, 5.4, 5.85, 7.2, 7.65, 8.1 and 9.45% NaCl [wt/vol]) (Table 5.11), but were not significantly different (t-test) (Table 5.15). Significant differences at pH 5, as revealed by t-tests, were at salinities 1, 2.25, 2.7, 3.15, 3.6 and 4.05% NaCl [wt/vol] (Table 5.15), where higher yields of aerobic growth are measured (1, 2.25, 2.7, 3.15, 3.6, 4.05% NaCl [wt/vol]) (Table 5.11). Mean max OD_{600} values obtained under pH 5 conditions at 30°C displayed highly significant strong negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.789, $p < 0.001$ and r = -0.788, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient) (Figure 5.6).

pH 4: Under pH 4 conditions, anaerobic growth prevailed over aerobic growth where yields were measured at 1, 2.25, 2.7, 3.15, 3.6, 4.05, 4.5, 4.95, 5.4 and 7.65% NaCl [wt/vol] for anaerobic cultures and only 0.93% NaCl [wt/vol] for aerobic cultures (Table 5.11). The only instance of growth under both aerobic and anaerobic conditions at pH 4 was statistically significantly different (1% NaCl [wt/vol]) (Welchs t-test) (Table 5.15). Mean max OD_{600} values obtained under pH 4 conditions at 30°C displayed highly significant strong negative linear correlation under anaerobic conditions (r = -0.71, $p < 0.001$; Pearsons product-moment correlation coefficient) (Figure 5.7).

35°C

At 35°C, a similar trend is seen as that at 30°C as aerobic growth of *E. coli* is higher than anaerobic growth at the lower range of salinities tested in this study under pH 7, 6 and 5 culture conditions.

pH 7: Higher mean max OD_{600} values were observed for 1, 2.25, 2.7, 3.15, 3.6, 4.05 and 4.5% NaCl [wt/vol] under aerobic conditions at pH 7 (Table 5.12), however two-sample independent Students t-test revealed no significant

differences in mean max OD_{600} values between aerobic and anaerobic conditions (Table 5.16). Higher mean max OD_{600} values were observed under anaerobic conditions for higher salinities at pH 7 (4.5, 4.95, 5.4, 5.85, 6.3, 6.75, 7.2, 7.65, 8.1 and 9.45% NaCl [wt/vol]) (Table 5.12). *T*-tests show these differences were not significant (Table 5.16). At pH 7 and 35°C, mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.930, $p < 0.001$ and r = -0.946, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient) (Figure 5.4).

pH 6: At pH 6, higher mean max OD_{600} values were observed for aerobic cultures than anaerobic cultures at lower salinities (1, 2.25, 2.7, 3.15, 3.6, 4.05, 4.5, 4.95 and 5.4% NaCl [wt/vol] (Table 5.12), with significant differences between culture conditions at 1, 3.6, 4.05 and 4.5% NaCl [wt/vol] (*t*-test) (Table 5.16). Higher mean max OD_{600} values were observed under anaerobic conditions than for aerobic conditions at pH 6 for higher salinities (5.85, 6.3, 6.75, 7.2, 7.65, 8.1, 8.55, 9, 9.45 and 9.9% NaCl [wt/vol](Table 5.12). However, *t*-tests show these differences were not significant (Table 5.16). At pH 6 and 35°C , mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.884, $p < 0.001$ and r = -0.929, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient) (Figure 5.5).

pH 5: At pH 5, higher mean max OD_{600} values were observed for aerobic cultures than anaerobic cultures at lower salinities (1, 2.25, 2.7, 3.15% NaCl [wt/vol] and for anaerobic cultures at higher salinities (4.5, 4.95, 5.4, 5.85, 7.2, 8.1, 8.55, 9, 9.45 and 9.9% NaCl [wt/vol] (Table 5.12). However, *t*-tests show these differences were not significant (Table 5.16). Higher mean max OD_{600} values were observed under anaerobic conditions than for aerobic conditions at pH 5 for 4.5, 4.95, 5.4, 5.85, 7.2, 8.1, 8.55, 9, 9.45 and 9.9% NaCl [wt/vol] (Table 5.12). *T*-tests reveal significant differences at salinities 1, 2.25, 2.7, 4.05 and 5.4% NaCl [wt/vol] (Table 5.16). At pH 5 and 35°C , mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.798, $p < 0.001$ and r = -0.734, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient) (Figure 5.6).

pH 4: Under pH 4 conditions, there are only two occasions in which growth

was observed for aerobic and anaerobic conditions (1% and 9% NaCl [wt/vol] respectively) (Table 5.12).

40°C

pH 7: At 40°C, higher mean max OD_{600} values were observed for 1, 2.25, 2.7, 3.15, 3.6, 4.05, 4.5, 4.95 and 5.4% NaCl [wt/vol] under aerobic conditions than anaerobic conditions at pH 7 (Table 5.13), and two-sample independent Students t-test revealed significant differences in mean max OD_{600} values between aerobic and anaerobic conditions at 2.25% NaCl [wt/vol] (Table 5.17). When cultured anaerobically, *E. coli* displayed higher growth under higher salinities at pH 7 with higher mean max OD_{600} values observed for 5.85, 6.3, 6.75, 7.2, 7.65, 8.1, 8.55, 9, 8.45 and 9.9% NaCl [wt/vol] (Table 5.13), however, t-tests revealed no significant differences between comparable yields (Table 5.17). At pH 7 and 40°C, mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.833, $p < 0.001$ and r = -0.771, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient) (Figure 5.4).

pH 6 nd 5: At pH 6 and 5, differences in mean max OD_{600} values displayed less of a trend throughout the salinity range tested in this study, with groupings of higher values obtained under aerobic conditions at lower salinities (1, 2.25, 2.7 and 3.15% NaCl [wt/vol] for both), and anaerobic conditions at higher salinities (8.1, 8.55, 9, 9.45 and 9.9% NaCl [wt/vol] for pH 6, and 8.55, 9, 9.45 and 9.9 NaCl [wt/vol] for pH 5) (Table 5.13). T-test revealed no significant differences in mean max OD_{600} values between aerobic and anaerobic conditions under pH 6 conditions and significant differences at salinities 1, 2.25, 2.7, 4.05, 4.5 and 5.4% NaCl [wt/vol] at pH 5 (Table 5.17).

At pH 6 and 40°C , mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.87, $p < 0.001$ and r = -0.877, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient) (Figure 5.5). At pH 5 and 40°C , mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.795, $p < 0.001$ and r = -0.795, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient) (Figure 5.6).

pH 4: Under pH 4 conditions, there are more instances of anaerobic growth

than under aerobic conditions throughout the salinities tested, with only one statistically significant difference (2.25% NaCl [wt/vol]) (Welchs t-test). At pH 4 and 40°C, mean max OD_{600} values displayed significant moderate negative linear correlation under anaerobic conditions (r = -0.475, $p < 0.05$; Pearsons product-moment correlation coefficient) (Figure 5.7).

45°C

pH 7: At 45°C, *E.coli* displayed higher growth under aerobic conditions than anaerobic conditions at pH 7 throughout the range of salinities tested in this study. Higher mean max OD_{600} values at all salinities excluding 2.25, 2.7, 5.85 and 9.9% NaCl [wt/vol] where anaerobic cultures displayed higher growth, and 8.55, 9 and 9.45% NaCl [wt/vol] where no growth was observed for either condition (Table 5.14). T-tests revealed significant differences between aerobic and anaerobic growth at 2.7% NaCl [wt/vol] at pH 7 (Table 5.18). At pH 7 and 45/*degree*C, mean OD_{600} values displayed non-significant moderate negative linear correlation with salinity under aerobic conditions (r = -0.445, p = 0.056; Pearsons product-moment correlation coefficient), and significant moderate negative linear correlation under anaerobic conditions (r = -0.558, $p < 0.05$; Pearsons product-moment correlation coefficient) (Figure 5.4).

pH 6: At pH 6, differences in mean max OD_{600} values were scattered throughout the salinities, with some clustering of higher anaerobic growth at salinities 2.25, 2.7, 3.15, 3.6 and 4.05% NaCl [wt/vol], however non of these instances was shown to be significantly different within this range (two-sample independent Students t-test). No significant differences between culture conditions were measured at pH 6 by t-test (Table 5.18). At pH 6 and 45°C , mean max OD_{600} values displayed significant moderate negative linear correlation with salinity under both aerobic conditions (r = -0.464, $p < 0.05$; Pearsons product-moment correlation coefficient), and highly significant moderate linear correlation under anaerobic conditions (r = -0.614, $p < 0.01$; Pearsons product-moment correlation coefficient) (Figure 5.5).

pH 5: At pH 5, aerobic growth was consistently higher than anaerobic growth throughout the range of salinities, with instances of no aerobic growth occurring at 6.75, 7.2, 7.65, 8.55, 9, 9.45 and 9.9% NaCl [wt/vol] and four instances of no anaerobic growth at 2.25, 3.15, 4.05 and 4.5% NaCl [wt/vol] (Table 5.14).

T-tests revealed significant differences between culture conditions at 1, 2.7 and 4.95% NaCl [wt/vol] (Table 5.18). At pH 5 and 45°C , mean max OD_{600} values were not correlated with salinity under aerobic or anaerobic conditions (Pearsons product-moment correlation coefficient) (Figure 5.6).

pH 4: At pH 4, there is limited growth under aerobic conditions with only two instances of measurable growth (4.95 and 9% NaCl [wt/vol]), with anaerobic cultures yielding viable growth under salinities 2.25, 3.15, 3.6, 4.05, 5.85, 6.3, 7.2, 7.65, 8.1, 8.55, 9 and 9.9% NaCl [wt/vol] (Table 5.14). Two-sample Students t-test revealed no significant difference between the only comparable growths (9% NaCl [wt/vol] (Table 5.18). At pH 4 and 45°C, mean max OD_{600} values were not correlated under anaerobic conditions ($r = 0.005$, $p = 982$; Pearsons product-moment correlation coefficient) (Figure 5.7).

Table (5.11) *Mean and Standard Deviation ± (n = 3) of the mean max OD_{600} values for* E.coli *cultured at 30°C under a range of pH values and salinity (% NaCl [wt/vol]) (n = 19).*

30°C	pH 7	pH 7	pH 6	pH 6	pH 5	pH 5	pH 4	pH 4
NaCl (%)	+	-	+	-	+	-	+	-
1	**1.693 ± 0.26**	0.855 ± 0.07	**1.924 ± 0.12**	1.155 ± 0.07	**1.913 ± 0.06**	0.769 ± 0.19	0.013 ± 0.001	**0.174 ± 0.03**
1.5	**1.679 ± 0.06**	0.895 ± 0.38	**2.491 ± 0.28**	1.370 ± 0.23	**1.862 ± 0.16**	0.641 ± 0.13	-	**0.080 ± 0.05**
2	**1.415 ± 0.32**	1.204 ± 0.51	**2.358 ± 0.65**	1.511 ± 0.29	**2.207 ± 0.10**	0.819 ± 0.44	-	**0.053 ± 0.01**
2.5	**1.201 ± 0.06**	0.966 ± 0.23	**2.515 ± 0.29**	1.340 ± 0.70	**1.995 ± 0.19**	0.792 ± 0.19	-	**0.049 ± 0.02**
3	**1.643 ± 0.6**	0.904 ± 0.46	**2.232 ± 0.38**	1.376 ± 0.61	**2.385 ± 0.54**	0.478 ± 0.10	-	**0.044 ± 0.02**
3.5	**1.181 ± 0.14**	0.969 ± 0.47	**2.425 ± 0.09**	0.983 ± 0.17	**2.231 ± 0.39**	0.815 ± 0.30	-	**0.019 ± 0.01**
4	**1.383 ± 0.11**	0.640 ± 0.22	**2.466 ± 0.06**	0.910 ± 0.20	-	**1.272 ± 0.87**	-	**0.017 ± 0.002**
4.5	**1.197 ± 0.32**	0.470 ± 0.05	**2.452 ± 0.2**	0.841 ± 0.55	-	**0.397 ± 0.25**	-	**0.016 ± 0.004**
5	**1.043 ± 0.24**	0.598 ± 0.01	**2.368 ± 0.43**	0.667 ± 0.20	0.016 ± 0.01	**0.814 ± 0.25**	-	**0.010 ± 0.003**
5.5	**0.522 ± 0.24**	0.498 ± 0.05	**1.863 ± 0.45**	0.565 ± 0.08	0.018 ± 0.002	**0.027 ± 0.01**	-	-
6	**0.461 ± 0.09**	0.284 ± 0.07	**0.362 ± 0.39**	0.215 ± 0.02	**0.013 ± 0.01**	-	-	-
6.5	**0.186 ± 0.12**	0.053 ± 0.01	0.021 ± 0.01	**0.092 ± 0.11**	-	-	-	-
7	0.022 ± 0.02	**0.082 ± 0.07**	0.029 ± 0.01	**0.050 ± 0.02**	-	**0.016 ± 0.001**	-	-
7.5	0.014 ± 0.01	0.014 ± 0.01	0.019 ± 0.01	**0.049 ± 0.05**	-	**0.018 ± 0.02**	-	**0.013 ± 0.01**
8	-	-	0.011 ± 0.01	**0.048 ± 0.03**	-	**0.030 ± 0.003**	-	-
8.5	-	-	-	**0.011 ± 0.003**	**0.011 ± 0.01**	-	-	-
9	-	**0.017 ± 0.01**	-	**0.039 ± 0.03**	-	-	-	-
9.5	-	**0.026 ± 0.02**	0.013 ± 0.01	**0.024 ± 0.01**	-	**0.011 ± 0.001**	-	-
10	-	**0.018 ± 0.01**	**0.013 ± 0.002**	0.012 ± 0.01	-	-	-	-

Table (5.12) *Mean and Standard Deviation ± (n = 3) of the mean max OD_{600} values for* E.coli *cultured at 35°C under a range of pH values and salinity (% NaCl [wt/vol]) (n = 19).*

35°C								
NaCl (%)	pH 7 +	pH 7 -	pH 6 +	pH 6 -	pH 5 +	pH 5 -	pH 4 +	pH 4 -
1	**1.775 ± 0.31**	1.236 ± 0.60	**1.814 ± 0.1**	1.074 ± 0.27	**2.020 ± 0.22**	0.765 ± 0.16	**0.020 ± 0.02**	-
1.5	**1.250 ± 0.14**	0.742 ± 0.12	**2.113 ± 0.59**	1.125 ± 0.48	**2.441 ± 0.35**	0.527 ± 0.03	-	-
2	**1.068 ± 0.06**	0.975 ± 0.36	**1.931 ± 0.62**	0.657 ± 0.29	**2.410 ± 0.20**	0.543 ± 0.03	-	-
2.5	**1.286 ± 0.07**	0.756 ± 0.17	**2.638 ± 0.17**	1.163 ± 0.66	**2.522 ± 0.14**	1.414 ± 0.88	-	-
3	**1.115 ± 0.06**	0.827 ± 0.14	**2.264 ± 0.21**	0.625 ± 0.10	-	0.550 ± 0.12	-	-
3.5	**0.894 ± 0.16**	0.730 ± 0.12	**2.328 ± 0.58**	0.773 ± 0.23	-	0.816 ± 0.32	-	-
4	-	**0.487 ± 0.01**	**2.350 ± 0.38**	0.725 ± 0.09	0.123 ± 0.18	**0.657 ± 0.30**	-	-
4.5	0.503 ± 0.2	**0.589 ± 0.13**	**1.676 ± 0.95**	0.620 ± 0.01	0.021 ± 0.03	**0.871 ± 0.39**	-	-
5	0.319 ± 0.03	**0.664 ± 0.34**	**1.174 ± 0.84**	0.680 ± 0.36	0.027 ± 0.01	**1.263 ± 0.33**	-	-
5.5	0.343 ± 0.07	**0.582 ± 0.32**	0.401 ± 0.03	**0.458 ± 0.02**	0.037 ± 0.02	**0.407 ± 0.34**	-	-
6	0.211 ± 0.12	**0.420 ± 0.25**	0.209 ± 0.08	**0.312 ± 0.08**	**0.011 ± 0.02**	-	-	-
6.5	0.099 ± 0.05	**0.141 ± 0.04**	0.041 ± 0.03	**0.090 ± 0.01**	-	-	-	-
7	0.023 ± <0.001	**0.106 ± 0.04**	0.031 ± 0.02	**0.032 ± 0.01**	-	**0.015 ± 0.004**	-	-
7.5	0.013 ± 0.01	**0.063 ± 0.08**	0.021 ± 0.01	**0.039 ± 0.01**	**0.016 ± 0.01**	-	-	-
8	0.012 ± 0.003	**0.030 ± 0.04**	0.021 ± 0.02	**0.035 ± 0.03**	-	**0.013 ± 0.0003**	-	-
8.5	-	-	-	**0.014 ± 0.01**	-	**0.017 ± 0.003**	-	-
9	-	-	0.012 ± 0.005	**0.013 ± 0.01**	-	**0.014 ± 0.003**	-	**0.084 ± 0.09**
9.5	-	**0.019 ± 0.02**	-	**0.043 ± 0.03**	-	**0.015 ± 0.001**	-	-
10	-	-	-	**0.011 ± 0.01**	-	**0.023 ± 0.02**	-	-

Table (5.13) *Mean and Standard Deviation ± (n = 3) of the mean max OD_{600} values for* E.coli *cultured at 40°C under a range of pH values and salinity (% NaCl [wt/vol]) (n = 19).*

40°C								
NaCl (%)	pH 7 +	pH 7 -	pH 6 +	pH 6 -	pH 5 +	pH 5 -	pH 4 +	pH 4 -
1	**2.062 ± 0.3**	1.704 ± 0.32	**1.924 ± 0.37**	1.290 ± 0.21	**1.967 ± 0.25**	0.708 ± 0.06	0.016 ± 0.003	0.016 ± 0.001
1.5	**1.307 ± 0.17**	0.561 ± 0.08	**1.509 ± 0.01**	1.397 ± 0.64	**1.389 ± 0.05**	0.531 ± 0.04	0.012 ± 0.002	**0.029 ± <0.001**
2	**1.189 ± 0.09**	0.625 ± 0.22	**1.351 ± 0.01**	0.972 ± 0.27	**1.117 ± 0.05**	0.689 ± 0.22	-	**0.020 ± 0.01**
2.5	**1.058 ± 0.10**	0.751 ± 0.26	**1.273 ± 0.02**	0.743 ± 0.36	**0.720 ± 0.10**	0.680 ± 0.55	-	-
3	**0.788 ± 0.02**	0.595 ± 0.13	-	**0.942 ± 0.23**	0.630 ± 0.08	**0.920 ± 0.17**	-	**0.011 ± 0.001**
3.5	**0.443 ± 0.02**	0.376 ± 0.05	**0.576 ± 0.02**	0.477 ± 0.05	0.472 ± 0.06	**1.377 ± 0.35**	-	**0.012 ± 0.004**
4	**0.223 ± 0.02**	0.185 ± 0.09	0.308 ± 0.09	**0.320 ± 0.13**	0.077 ± 0.06	**0.665 ± 0.03**	-	-
4.5	**0.226 ± 0.03**	0.145 ± 0.03	**0.359 ± 0.1**	0.208 ± 0.09	**0.033 ± 0.03**	-	-	-
5	**0.120 ± 0.03**	0.086 ± 0.02	0.157 ± 0.02	**0.436 ± 0.33**	0.061 ± 0.05	**0.457 ± 0.03**	0.010 ± 0.002	**0.015 ± 0.01**
5.5	0.070 ± 0.01	**0.073 ± 0.04**	**0.187 ± 0.03**	0.144 ± 0.06	0.137 ± 0.02	**0.158 ± 0.05**	-	**0.028 ± 0.01**
6	0.028 ± 0.01	**0.038 ± 0.04**	0.086 ± 0.05	**0.137 ± 0.06**	0.160 ± 0.04	**0.199 ± 0.19**	-	**0.012 ± 0.01**
6.5	0.021 ± 0.01	**0.034 ± 0.03**	0.018 ± 0.003	**0.025 ± 0.02**	**0.086 ± 0.06**	0.057 ± 0.05	-	-
7	-	**0.063 ± 0.03**	**0.055 ± 0.02**	0.023 ± 0.002	**0.083 ± 0.07**	0.050 ± 0.01	-	**0.021 ± 0.02**
7.5	-	**0.028 ± 0.02**	**0.034 ± 0.01**	0.032 ± 0.03	**0.083 ± 0.03**	0.038 ± 0.01	-	**0.012 ± 0.01**
8	-	**0.020 ± 0.01**	0.059 ± 0.01	**0.064 ± 0.04**	**0.055 ± 0.04**	0.046 ± 0.02	-	-
8.5	-	**0.022 ± 0.01**	0.030 ± 0.001	**0.063 ± 0.02**	-	**0.051 ± 0.04**	-	-
9	-	**0.019 ± 0.003**	-	**0.055 ± 0.04**	-	**0.041 ± 0.01**	**0.020 ± 0.01**	0.011 ± 0.001
9.5	-	**0.017 ± 0.001**	-	**0.039 ± 0.01**	-	**0.024 ± 0.02**	-	**0.010 ± 0.005**
10	-	**0.011 ± 0.005**	0.011 ± 0.01	**0.021 ± 0.004**	-	**0.018 ± 0.01**	**0.011 ± 0.0004**	-

Table (5.14) *Mean and Standard Deviation ± (n = 3) of the mean max OD_{600} values for* E.coli *cultured at 45°C under a range of pH values and salinity (% NaCl [wt/vol]) (n = 19).*

45°C NaCl (%)	pH 7 +	pH 7 -	pH 6 +	pH 6 -	pH 5 +	pH 5 -	pH 4 +	pH 4 -
1	**1.725 ± 0.17**	1.048 ± 0.24	**2.156 ± 0.15**	1.266 ± 0.37	**2.284 ± 0.19**	0.393 ± 0.11	-	-
1.5	0.169 ± 0.03	**0.302 ± 0.09**	0.405 ± 0.03	**0.706 ± 0.41**	**0.068 ± 0.01**	-	-	**0.011 ± 0.003**
2	0.086 ± 0.03	**0.297 ± 0.04**	0.074 ± 0.01	**0.359 ± 0.15**	**0.038 ± 0.005**	0.011 ± 0.002	-	-
2.5	**0.034 ± 0.01**	0.028 ± 0.03	0.022 ± 0.003	**0.032 ± 0.01**	**0.042 ± 0.01**	-	-	**0.103 ± 0.003**
3	**0.023 ± 0.01**	0.010 ± 0.01	0.026 ± 0.01	**0.056 ± 0.04**	**0.043 ± 0.01**	0.027 ± 0.02	-	**0.012 ± 0.14**
3.5	**0.025 ± 0.003**	0.023 ± 0.01	0.029 ± 0.01	**0.064 ± 0.03**	**0.037 ± 0.005**	-	-	**0.022 ± 0.01**
4	**0.031 ± 0.01**	0.012 ± 0.01	**0.045 ± 0.02**	0.034 ± 0.05	**0.036 ± 0.01**	-	-	-
4.5	**0.014 ± 0.01**	-	**0.029 ± 0.01**	0.012 ± 0.01	**0.041 ± 0.003**	0.014 ± 0.01	**0.011 ± 0.01**	-
5	**0.026 ± 0.01**	0.018 ± 0.02	0.019 ± 0.01	**0.052 ± 0.02**	**0.030 ± 0.01**	0.018 ± 0.01	-	-
5.5	0.015 ± 0.01	**0.043 ± 0.05**	**0.037 ± 0.01**	0.025 ± 0.01	**0.041 ± 0.01**	0.019 ± 0.02	-	**0.014 ± 0.005**
6	**0.029 ± 0.004**	0.013 ± 0.01	0.024 ± 0.004	**0.025 ± 0.01**	**0.030 ± 0.01**	0.015 ± 0.01	-	**0.017 ± 0.005**
6.5	**0.024 ± 0.02**	0.015 ± 0.01	0.025 ± 0.01	0.025 ± 0.01	-	**0.014 ± 0.01**	-	-
7	**0.014 ± 0.01**	-	0.016 ± 0.01	**0.023 ± 0.02**	-	**0.021 ± 0.01**	-	**0.028 ± 0.04**
7.5	**0.017 ± 0.002**	-	**0.020 ± 0.01**	0.012 ± 0.01	-	**0.014 ± 0.01**	-	**0.016 ± 0.01**
8	**0.012 ± 0.002**	-	**0.021 ± 0.02**	-	**0.015 ± 0.01**	0.014 ± 0.02	-	**0.017 ± 0.02**
8.5	-	-	-	-	-	-	-	**0.016 ± 0.01**
9	-	-	0.013 ± 0.001	0.013 ± 0.001	-	**0.012 ± 0.01**	0.010 ± 0.002	**0.013 ± 0.005**
9.5	-	-	-	**0.013 ± 0.01**	-	-	-	-
10	-	**0.010 ± 0.0001**	-	-	-	-	-	**0.024 ± 0.02**

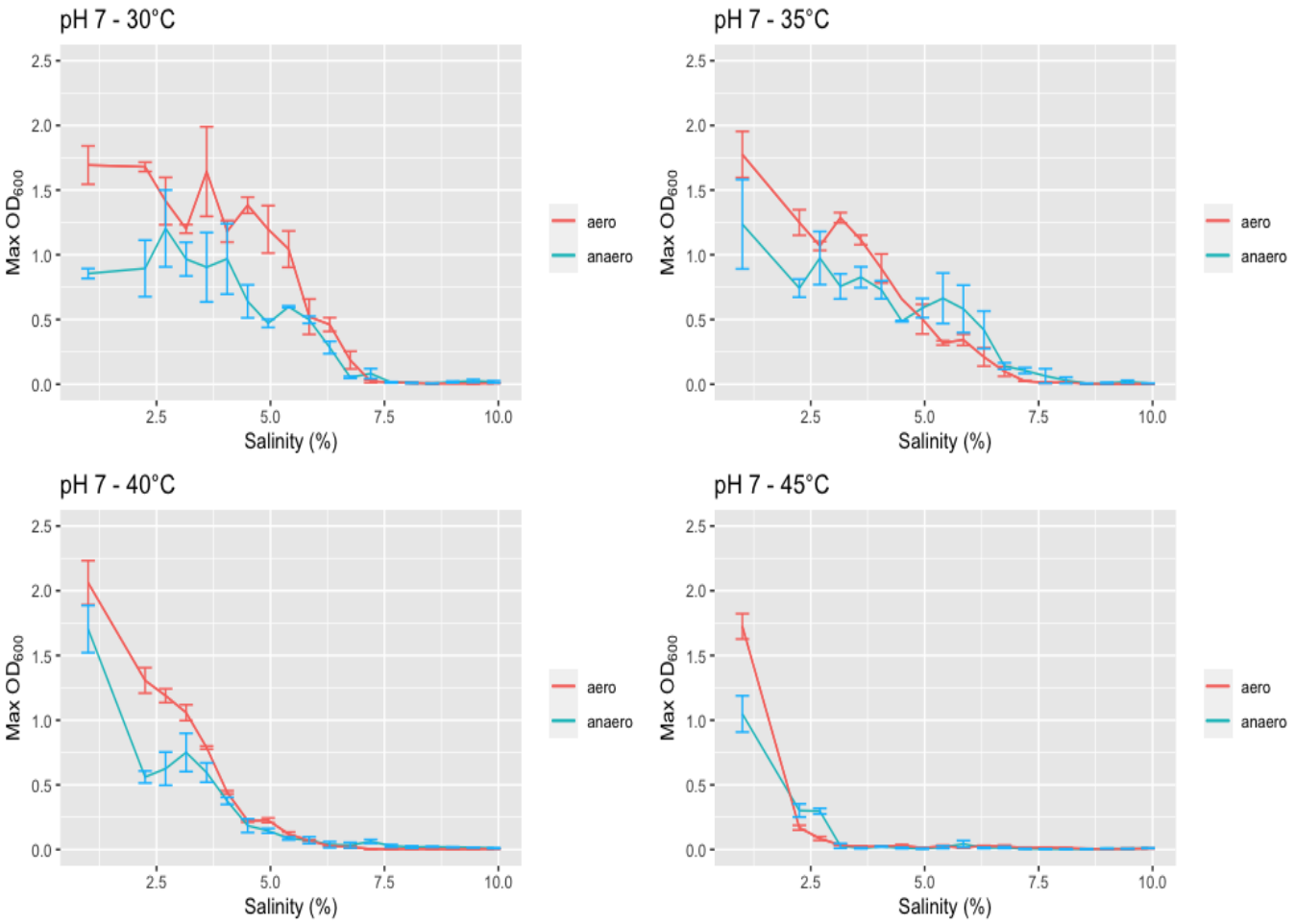

Figure (5.4) *Graph displaying mean maximal OD_{600} values obtained under pH 7 and a range of salinities (% NaCl [wt/vol] (n = 19), and temperatures for* E. coli. *Data presented as mean max OD_{600} values ± standard error of the means (SE) (n = 3).*

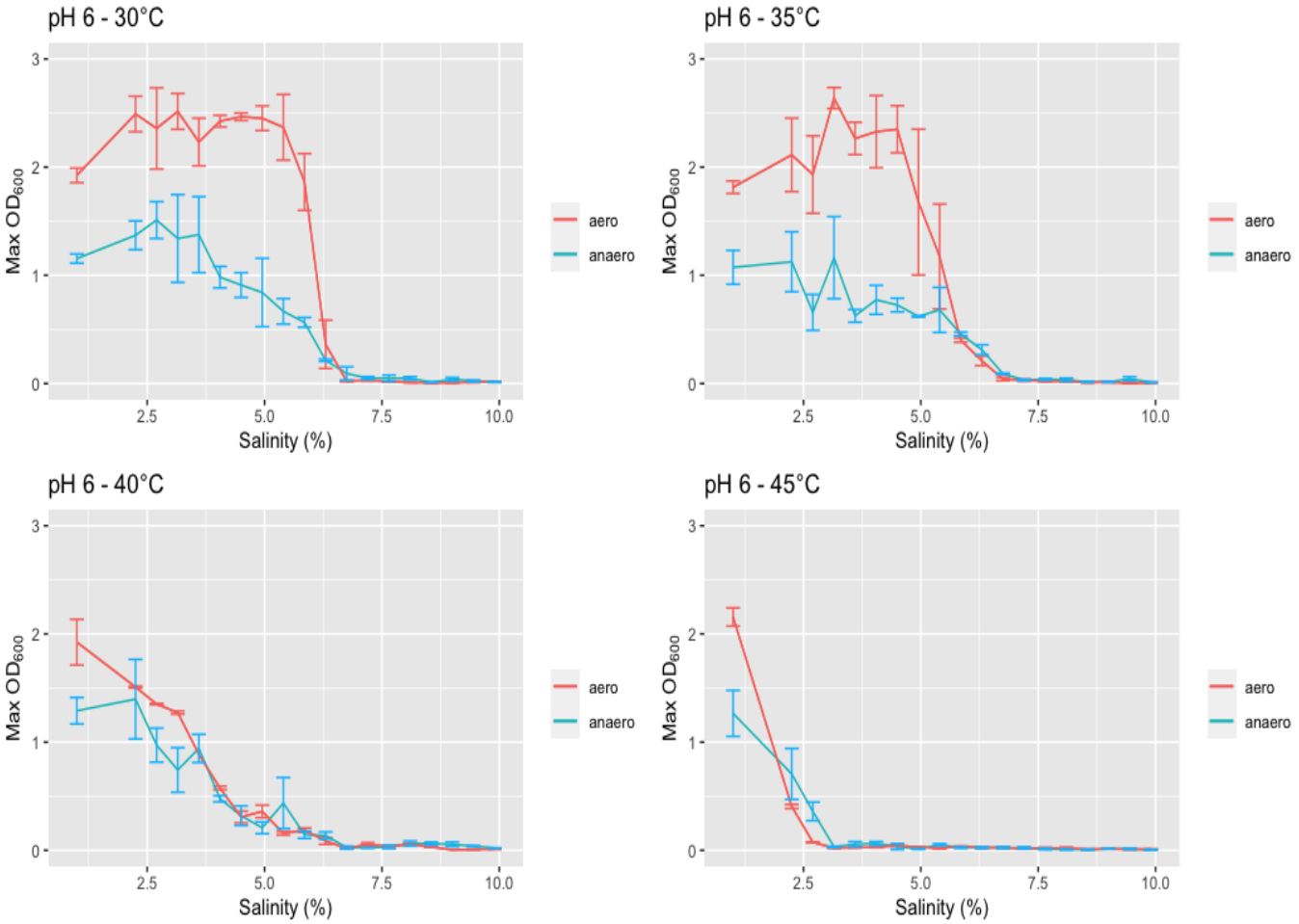

Figure (5.5) *Graph displaying mean maximal OD_{600} values obtained under pH 6 and a range of salinities (% NaCl [wt/vol] (n = 19), and temperatures for* E. coli. *Data presented as mean max OD_{600} values ± standard error of the means (SE) (n = 3).*

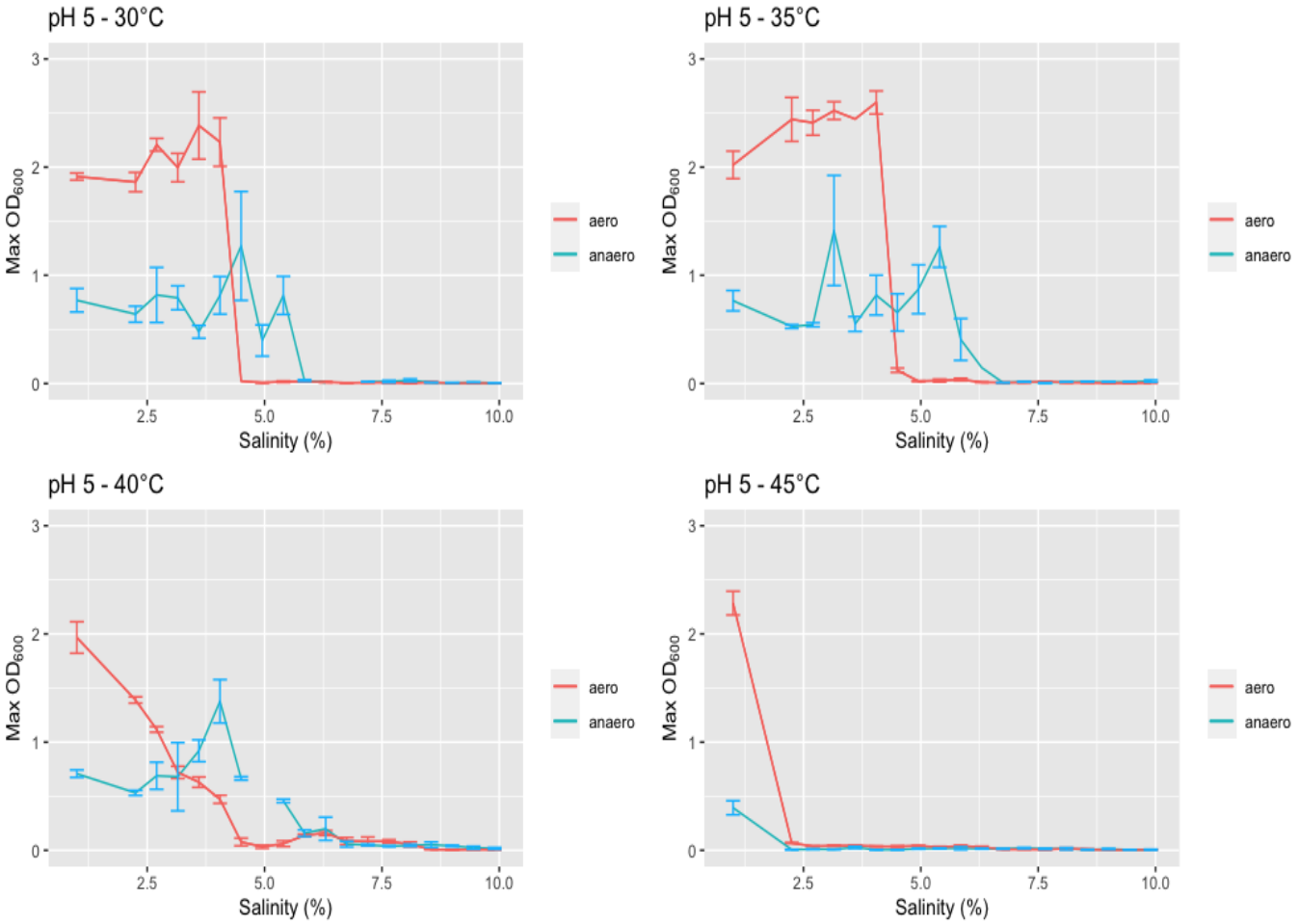

Figure (5.6) *Graph displaying mean maximal OD_{600} values obtained under pH 5 and a range of salinities (% NaCl [wt/vol] (n = 19), and temperatures for* E. coli. *Data presented as mean max OD_{600} values ± standard error of the means (SE) (n = 3).*

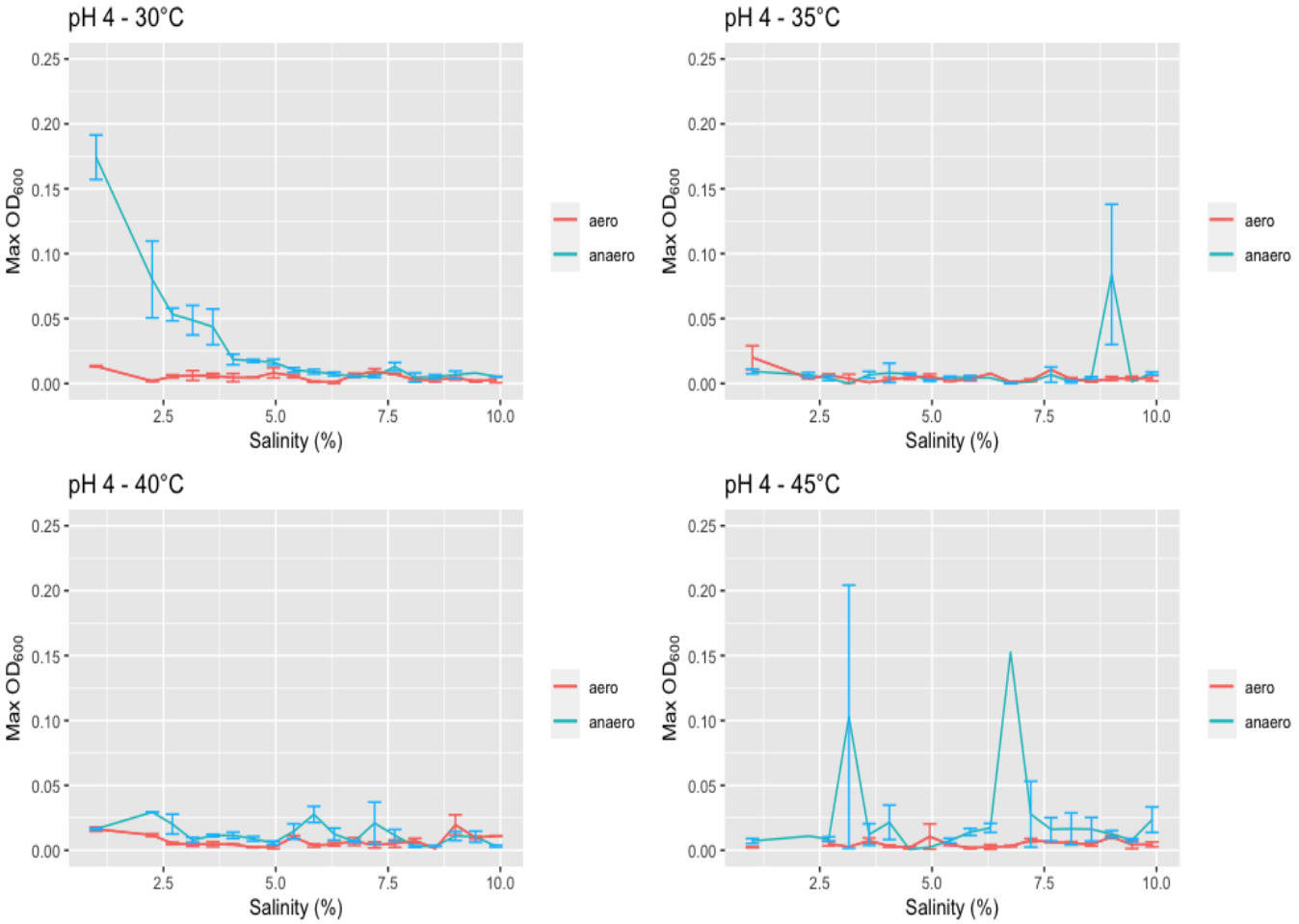

Figure (5.7) *Graph displaying mean maximal OD_{600} values obtained under pH 4 and a range of salinities (% NaCl [wt/vol] (n = 19), and temperatures for* E. coli. *Data presented as mean max OD_{600} values ± standard error of the means (SE) (n = 3).*

Table (5.15) *T-test results comparing aerobic and anaerobic maximal OD_{600} values obtained for* E. coli *cultured under a range of pH conditions and salinities (% NaCl [wt/vol]) (n = 19) at 30C.*
Green indicates instances in which neither condition yielded measurable data, red indicates no aerobic growth and blue indicates no anaerobic growth.
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

30°C NaCl (%)	pH 7	pH 6	pH 5	pH 4
1	$t(4) = 5.5$, **p <0.05**	$t(4) = 9.7$, **p <0.01**	$t(4) = 10.1$, **p <0.01**	$t(2) = -9.4$, **p <0.05***
2.25	$t(4) = 3.5$, p = 0.083	$t(4) = 5.3$, **p <0.05**	$t(4) = 10.5$, **p <0.01**	
2.7	$t(4) = 0.6$, $p = 0.672$	$t(4) = 2.1$, $p = 0.167$	$t(4) = 5.3$, **p <0.01**	
3.15	$t(4) = 1.7$, p = 0.291	$t(4) = 2.7$, $p = 0.117$	$t(3) = 6.9$, **p <0.01**	
3.6	$t(4) = 1.7$, $p = 0.291$	$t(4) = 2.1$, $p = 0.167$	$t(4) = 6$, **p <0.01***	
4.05	$t(4) = 0.7$, $p = 0.633$	$t(4) = 12.7$, **p <0.01**	$t(4) = 5$, **p <0.05***	
4.5	$t(4) = 5.2$, **p <0.05**	$t(4) = 13$, **p <0.01**		
4.95	$t(4) = 4$, $p = 0.816$	$t(4) = 4.8$, **p <0.05**		
5.4	$t(3) = 2.5$, $p = 0.247$	$t(3) = 6.2$, **p <0.05**	$t(1) = -4.5$, $p = 0.157$*	
5.85	$t(4) = 1.2$, $p = 0.940$	$t(4) = 4.9$, **p <0.05**	$t(3) = -1.4$, $p = 0.252$*	
6.3	$t(3) = 2.3$, $p = 0.247$	$t(2) = 0.7$, $p = 0.612$*		
6.75	$t(3) = 1.5$, $p = 0.319$	$t(2) = -1.1$, $p = 0.421$*		
7.2	$t(4) = -1.5$, $p = 0.318$	$t(4) = -1.3$, $p = 0.356$		
7.65	$t(4) = 0.05$, $p = 0.959$	$t(4) = -1$, $p = 0.421$		
8.1		$t(4) = -2.4$, $p = 0.136$		
8.55				
9				
9.45		$t(4) = 1.3$, $p = 0.357$		
9.9		$t(4) = 0.2$, $p = 0.825$		

Table (5.16) *T-test results comparing aerobic and anaerobic maximal OD_{600} values obtained for* E. coli *cultured under a range of pH conditions and salinities (% NaCl [wt/vol]) (n = 19) at 35C.*
Green indicates instances in which neither condition yielded measurable data, red indicates no aerobic growth and blue indicates no anaerobic growth.
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

35°C NaCl (%)	pH 7	pH 6	pH 5	pH 4
1	$t(4) = 1.4, p = 0.424$	$t(4) = 4.4, \mathbf{p < 0.05}$	$t(4) = 7.9, \mathbf{p < 0.01}$*	
2.25	$t(3) = 4.4, p = 0.148$	$t(4) = 2.3, p = 0.174$	$t(2) = 9.3, \mathbf{p < 0.05}$	
2.7	$t(4) = 0.5, p = 0.675$	$t(4) = 3.2, p = 0.085$	$t(4) = 16, \mathbf{p < 0.001}$	
3.15	$t(4) = 5.1, p = 0.099$	$t(4) = 3.8, p = 0.063$	$t(4) = 2.1, p = 0.110$	
3.6	$t(4) = 3.2, p = 0.148$	$t(3) = 12.2, \mathbf{p < 0.05}$		
4.05	$t(3) = 1.3, p = 0.423$	$t(4) = 4.3, \mathbf{p < 0.05}$	$t(4) = 8.4, \mathbf{p < 0.01}$	
4.5		$t(3) = 5.7, \mathbf{p < 0.05}$	$t(2) = -3.1, p = 0.110$*	
4.95	$t(4) = -0.6, p = 0.606$	$t(1) = 1.6, p = 0.482$*	$t(2) = -3.8, p = 0.095$*	
5.4	$t(2) = -1.8, p = 0.424$*	$t(4) = 0.9, p = 0.495$	$t(2) = -6.5, \mathbf{p < 0.05}$*	
5.85	$t(4) = -1.3, p = 0.273$	$t(3) = -2, p = 0.216$	$t(2) = -1.9, p = 0.196$*	
6.3	$t(4) = -1.3, p = 0.422$	$t(4) = -1.6, p = 0.265$		
6.75	$t(3) = -0.1, p = 0.558$	$t(4) = -2.9, p = 0.098$		
7.2	$t(2) = -3.7, p = 0.234$*	$t(4) = -0.1, p = 0.904$		
7.65	$t(1) = -0.9, p = 0.606$*	$t(4) = -2, p = 0.212$		
8.1	$t(2) = -0.7, p = 0.537$*	$t(4) = -0.7, p = 0.583$		
8.55				
9		$t(4) = -0.3, p = 0.834$		
9.45				
9.9				

Table (5.17) *T-test results comparing aerobic and anaerobic maximal OD_{600} values obtained for* E. coli *cultured under a range of pH conditions and salinities (% NaCl [wt/vol]) (n = 19) at 40C.*
Green indicates instances in which neither condition yielded measurable data, red indicates no aerobic growth and blue indicates no anaerobic growth.
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

40°C NaCl (%)	pH 7	pH 6	pH 5	pH 4
1	$t(4) = 1.4, p = 0.337$	$t(4) = 2.6, p = 0.338$	$t(4) = 8.4, \mathbf{p < 0.01}$	$t(4) = -0.07, p = 0.949$
2.25	$t(4) = 6.9, \mathbf{p < 0.05}$	$t(2) = 0.3, p = 0.905$*	$t(4) = 23, \mathbf{p < 0.001}$	$t(2) = -16.6, \mathbf{p < 0.05}$*
2.7	$t(4) = 4.1, p = 0.923$	$t(2) = 2.4, p = 0.338$*	$t(4) = 3.4, \mathbf{p < 0.05}$	
3.15	$t(4) = 1.9, p = 0.283$	$t(2) = 2.6, p = 0.339$*	$t(4) = 0.1, p = 0.905$	
3.6	$t(3) = 2, p = 0.284$		$t(4) = -2.6, p = 0.105$	
4.05	$t(4) = 2.2, p = 0.277$	$t(3) = 2.5, p = 0.338$	$t(4) = -4.4, \mathbf{p < 0.05}$	
4.5	$t(4) = 0.7, p = 0.702$	$t(3) = -0.1, p = 0.906$	$t(4) = -15, \mathbf{p < 0.001}$	
4.95	$t(4) = 3.2, p = 0.133$	$t(4) = 1.9, p = 0.131$		
5.4	$t(4) = 1.7, p = 0.284$	$t(2) = -1.2, p = 0.521$	$t(4) = -12.7, \mathbf{p < 0.01}$	$t(2) = -0.7, p = 0.710$
5.85	$t(3) = -0.1, p = 0.916$	$t(4) = 1.1, p = 0.330$	$t(4) = -0.6, p = 0.717$	
6.3	$t(4) = -0.4, p = 0.784$	$t(4) = -1.1, p = 0.522$	$t(4) = -0.4, p = 0.796$	
6.75	$t(2) = -0.6, p = 0.759$	$t(2) = -0.5, p = 0.877$*	$t(4) = 0.7, p = 0.717$	
7.2		$t(1) = 2, p = 0.521$*	$t(4) = 0.8, p = 0.716$	
7.65		$t(4) = 0.2, p = 0.905$	$t(4) = 2.8, p = 0.094$	
8.1		$t(4) = -0.2, p = 0.905$	$t(4) = 0.4, p = 0.776$	
8.55		$t(3) = -1.9, p = 0.338$		
9				$t(3) = 0.08, p = 0.465$
9.45				
9.9		$t(4) = -2.3, p = 0.349$		

Table (5.18) *T-test results comparing aerobic and anaerobic maximal OD_{600} values obtained for* E. coli *cultured under a range of pH conditions and salinities (% NaCl [wt/vol]) (n = 19) at 45C.*
Green indicates instances in which neither condition yielded measurable data, red indicates no aerobic growth and blue indicates no anaerobic growth.
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

45°C NaCl (%)	pH 7	pH 6	pH 5	pH 4
1	$t(4) = 4, p = 0.092$	$t(4) = 3.9, p = 0.132$	$t(4) = 14.8, \mathbf{p < 0.001}$	
2.25	$t(4) = -2.5, p = 0.189$	$t(2) = -1.3, p = 0.548$*		
2.7	$t(4) = -8, \mathbf{p < 0.05}$	$t(2) = -3.3, p = 0.296$*	$t(4) = 9.2, \mathbf{p < 0.01}$	
3.15	$t(4) = 0.3, p = 0.773$	$t(4) = -1.6, p = 0.395$		
3.6	$t(4) = 1.8, p = 0.280$	$t(4) = -1.3, p = 0.520$	$t(4) = 1.4, p = 0.268$	
4.05	$t(4) = 0.5, p = 0.689$	$t(4) = -2, p = 0.342$		
4.5	$t(4) = 2, p = 0.262$	$t(4) = 0.4, p = 0.849$		
4.95		$t(4) = 4, p = 0.132$	$t(4) = 6.5, \mathbf{p < 0.01}$	
5.4	$t(4) = 0.7, p = 0.667$	$t(4) = -3.3, p = 0.144$	$t(4) = 1.5, p = 0.219$	
5.85	$t(2) = -1.1, p = 0.603$*	$t(4) = 1.6, p = 0.395$	$t(4) = 1.4, p = 0.235$	
6.3	$t(4) = 3.1, p = 0.129$	$t(4) = -0.2, p = 0.945$	$\lvert t \rvert(4) = 1.8, p = 0.268$	
6.75	$t(4) = 0.7, p = 0.667$	$t(4) = 0.06, p = 0.958$		
7.2		$t(4) = -0.6, p = 0.726$		
7.65		$t(4) = 0.9, p = 0.641$		
8.1			$t(3) = 0.07, p = 0.947$	
8.55				
9		$t(4) = -0.7, p = 0.536$		$t(4) = -0.8, p = 0.491$
9.45				
9.9				

5.4.3 Aerobic vs Anaerobic Conditions for Carnobacterium pleistocenium Under the Combined Stress of Salinity, pH and Supra-optimal Temperatures.

To measure the relationship between concentrations of NaCl [wt/vol], variations in pH and temperature on microbial propagation employing different modes of metabolism, *Carnobacterium pleistocenium* was cultured under a range of salinities, pH and temperatures under aerobic conditions and anaerobic conditions.

24°C

When cultured aerobically, *C. pleistocenium* displayed higher growth at 24°C at the lower salinity range under pH 7.5 than anaerobically. Higher mean max OD_{600} values were observed under aerobic conditions for salinities 0, 0.5, 1, 1.5 and 2% NaCl [wt/vol] (Table 5.19). *T*-tests revealed a significant difference between conditions at 1.5% NaCl [wt/vol] (Table 5.24), with no measurable anaerobic growth occurring at 0, 1 and 1.5% NaCl [wt/vol]. Higher mean max OD_{600} values were observed under anaerobic conditions than at aerobic conditions at higher NaCl concentrations at pH 7.5 (2.5, 3, 3.5, 4, 4.5 and 5% NaCl [wt/vol]) (Table 5.19) with no measurable aerobic growth at 3.5, 4.5 and 5% NaCl [wt/vol] (Table 5.24). Mean max OD_{600} values obtained under pH 7 conditions were higher under aerobic conditions than anaerobic conditions at salinities 0, 0.5, 1, 1.5, 2, 2.5, 3 and 4% NaCl [wt/vol], and anaerobic growth higher at salinities 3.5 and 4.5% NaCl [wt/vol] (Table 5.19). There were no instances of measurable growth for anaerobic cultures growth at 24°C and pH 6.5 and pH 6 (Table 5.19). Mean max OD_{600} values obtained under pH 7.5 conditions at 24°C displayed highly significant strong negative linear correlation with salinity under aerobic conditions (r = -0.892, $p < 0.01$; Pearsons product-moment correlation coefficient), and were not significantly correlated under anaerobic conditions (r = -0.073, p = 0.841; Pearsons product-moment correlation coefficient) (Figure 5.8). At pH 7, mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under aerobic conditions (r = -0.961, $p < 0.001$; Pearsons product-moment correlation coefficient), and were not significantly correlated under anaerobic conditions (r = 0.128, p = 0.762; Pearsons product-moment correlation coefficient) (Figure 5.9). At pH 6.5, mean max OD_{600} values displayed

significant strong negative linear correlation with salinity under aerobic conditions (r = -0.752, $p < 0.05$; Pearsons product-moment correlation coefficient), and no testable growth was detected under anaerobic conditions (Figure 5.10). At pH 6 conditions, mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under aerobic conditions (r = -0.867, $p < 0.001$; Pearsons product-moment correlation coefficient), and no testable growth was detected under anaerobic conditions (Figure 5.11).

26°C

At 26°C, *C. pleistocenium* displayed higher growth at the lower salinities tested (0 and 0.5% NaCl [w/vol] under aerobic conditions than anaerobic conditions, with one incident of higher anaerobic growth (4.5% NaCl [wt/vol]) (Table 5.20). Two-sample independent Students t-tests revealed a significant difference between conditions at 0.5% NaCl [wt/vol] (Table 5.24). There were no instances of measurable growth for anaerobic cultures growth at 26°C and pH 7, 6.5 and pH 6 (Table 5.20). At pH 7.5, mean max OD_{600} values displayed non-significant strong negative linear correlation with salinity under aerobic conditions (r = -0.864, $p = 0.263$; Pearsons product-moment correlation coefficient) (Figure 5.8). At pH 7, mean max OD_{600} values displayed significant strong negative linear correlation with salinity under aerobic conditions (r = -0.833, $p < 0.05$; Pearsons product-moment correlation coefficient) (Figure 5.9). At pH 6.5 and 26°C, mean OD_{600} values displayed highly significant strong negative linear correlation with salinity under aerobic conditions (r = -0.936, $p < 0.001$; Pearsons product-moment correlation coefficient) (Figure 5.10). At pH 6, mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under aerobic conditions (r = -0.938, $p < 0.001$; Pearsons product-moment correlation coefficient) (Figure 5.11).

28°C

At 28°C, *C. pleistocenium* displayed increased yields under anaerobic conditions at higher salinities when compared with aerobic growth. At pH 7.5, higher mean max OD_{600} values were observed under aerobic conditions for salinities 0, 0.5, 1, 1.5 and 2% NaCl [wt/vol] after which no aerobic growth was observed (Table 5.21). T-tests revealed significant differences in mean max OD_{600} values

at 0 and 0.5% NaCl [wt/vol] (Table 25). Higher mean max OD_{600} values were observed under anaerobic conditions for higher salinities at pH 7.5 (2.5, 3, 3.5, 4, 4.5 and 5% NaCl [wt/vol]). At pH 7, aerobic cultures yielded higher mean max OD_{600} values than anaerobic cultures at lower salinities (0, 0.5, 1, 1.5, 2, 2.5 and 3% NaCl [wt/vol] (Table 5.21), with significant differences at 0, 0.5, 1 and 2% NaCl [wt/vol] (t-test) (Table 5.25). Higher mean max OD_{600} values were observed under anaerobic conditions than aerobic conditions for higher salinities at pH 7 (3.5, 4, 4.5 and 5% NaCl wt/vol]) (Table 5.21), with the only significant difference at 4% NaCl [wt/vol] (t-test) (Table 5.25). At pH 6.5, higher mean max OD_{600} values were observed under aerobic conditions than anaerobic conditions for salinities 0, 1, 1.5, 2 and 2.5% NaCl [wt/vol], and higher mean max OD_{600} values were observed under anaerobic conditions at higher salinities (3, 3.5, 4, 4.5 and 5% NaCl [wt/vol]) (Table 5.21). Statistically significant differences, as revealed by t-tests, are at 0, 2.5, 5 and 4.5% NaCl [wt/vol] (Table 5.25). At pH 6, mean max OD_{600} values displayed less of a pattern, however some clustering of higher yields are seen at lower salinities under aerobic conditions (0, 0.5 and 1% NaCl [wt/vol]) and at the higher salinities for anaerobic growth (2.5, 3, 3.5, 4.5 and 5% NaCl [wt/vol]) (Table 5.21). T-tests revealed significant differences at 0, 1 and 3% NaCl [wt/vol] (Table 5.25). At pH 7.5 and 28°C, mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under aerobic conditions (r = -0.989, $p < 0.01$; Pearsons product-moment correlation coefficient), and non-significant moderate negative linear correlation under anaerobic conditions (r = -0.498, p = 0.119; Pearsons product-moment correlation coefficient) (Figure 5.8). At pH 7, mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under aerobic conditions (r = -0.991, $p < 0.001$; Pearsons product-moment correlation coefficient), and non-significant moderate negative linear correlation under anaerobic conditions (r = -0.344, p = 0.3; Pearsons product-moment correlation coefficient) (Figure 5.9). At pH 6.5, mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under aerobic conditions (r = -0.968, $p < 0.001$; Pearsons product-moment correlation coefficient), and non-significant weak negative correlation under anaerobic conditions (r = -0.274, p = 0.416; Pearsons product-moment correlation coefficient) (Figure 5.10). At pH 6, mean max OD_{600} values displayed highly significant strong negative linear correlation with salinity under aerobic conditions (r = -0.809, $p < 0.01$; Pearsons product-moment correlation coefficient), and were not significantly correlated under anaerobic conditions (r

= -0.039, p = 0.908; Pearsons product-moment correlation coefficient) (Figure 5.11).

30°C

At 30°C, *C. pleistocenium* yielded measurable aerobic growth under pH 7.5 conditions for the two lowest salinities tested (0 and 0.5% NaCl [wt/vol]), and anaerobic growth at 1, 2, 2.5 and 3% NaCl [wt/vol] (Table 5.22). At pH 7, only one instance of aerobic growth occurred (0% NaCl [wt/vol]) and there were no instances of anaerobic growth (Table 5.22). At pH 6.5, anaerobic growth was higher than aerobic growth for all salinities excluding 0 and 1.5% NaCl [wt/vol], with 1.5% being statistically significantly different (t-test) (Table 5.26). The only other significantly difference, as revealed by t-test, was at 2% NaCl [wt/vol] for pH 6.5 (Table 5.26). At pH 6, anaerobic growth was higher than aerobic growth for all salinities excluding only 0% NaCl [wt/vol] (Table 5.22), however, t-tests revealed no significant differences. At pH 7.5 and 30°C, mean max OD_{600} values displayed non-significant weak negative linear correlation under anaerobic conditions (r = -0.26, p = 0.534; Pearsons product-moment correlation coefficient) (Figure 5.8). At pH 6.5, mean max OD_{600} values displayed non significant linear correlation with salinity under aerobic conditions (r = -0.039, $p < 0.94$; Pearsons product-moment correlation coefficient), and non-significant weak positive linear correlation under anaerobic conditions (r = 0.215, p = 0.551; Pearsons product-moment correlation coefficient) (Figure 5.10). At pH 6, mean max OD_{600} values displayed non-significant moderate negative linear correlation with salinity under both aerobic and anaerobic conditions (r = -0.416, p = 0.412 and r = -0.396, p = 0.228 respectively; Pearsons product-moment correlation coefficient).

Table (5.19) *Mean and Standard Deviation ± (n = 3) of the mean max OD_{600} values for* C. pleistocenium *cultured at 24°C under a range of pH values and salinity (% NaCl [wt/vol]) (n = 11).*

24°C	pH 7.5	pH 7.5	pH 7	pH 7	pH 6.5	pH 6.5	pH 6	pH 6
NaCl (%)	+	-	+	-	+	-	+	-
0	**1.405 ± 0.1**	-	**1.425 ± 0.07**	-	**1.225 ± 0.6**	-	**1.414 ± 0.02**	-
0.5	**1.21 ± 0.23**	-	**1.373 ± 0.02**	-	**1.472 ± 0.06**	-	**1.411 ± 0.07**	-
1	**1.465 ± 0.04**	-	**1.326 ± 0.02**	-	**1.127 ± 0.7**	-	**1.434 ± 0.02**	-
1.5	**1.315 ± 0.08**	0.012 ± 0.003	**1.317 ± 0.1**	0.016 ± 0.002	**1.229 ± 0.33**	-	**1.436 ± 0.07**	-
2	**0.305 ± 0.44**	0.036 ± 0.02	**1.041 ± 0.23**	0.018 ± 0.001	**1.431 ± 0.04**	-	**1.425 ± 0.04**	-
2.5	0.011 ± 0.002	**0.019 ± 0.005**	**0.631 ± 0.02**	-	**1.432 ± 0.03**	-	**1.316 ± 0.09**	-
3	0.011 ± 0.001	**0.016 ± 0.003**	**0.507 ± 0.3**	0.011 ± 0.0003	**1.214 ± 0.06**	-	**1.121 ± 0.08**	-
3.5	-	**0.015 ± 0.002**	0.136 ± 0.19	**0.441 ± 0.6**	**0.716 ± 0.05**	-	**1.014 ± 0.13**	-
4	0.015 ± 0.003	**0.041 ± 0.015**	**0.041 ± 0.05**	0.023 ± 0.002	**0.660 ± 0.4**	-	**0.933 ± 0.17**	-
4.5	-	**0.017 ± 0.003**	-	**0.012 ± 0.002**	**0.156 ± 0.03**	-	**0.326 ± 0.22**	-
5	-	**0.015 ± 0.002**	-	-	-	-	**0.052 ± 0.07**	-

Table (5.20) *Mean and Standard Deviation ± (n = 3) of the mean max OD_{600} values for* C. pleistocenium *cultured at 26°C under a range of pH values and salinity (% NaCl [wt/vol]) (n = 11).*

26°C	pH 7.5	pH 7.5	pH 7	pH 7	pH 6.5	pH 6.5	pH 6	pH 6
NaCl (%)	+	-	+	-	+	-	+	-
0	1.135 ± 0.44	0.142 ± 0.18	1.346 ± 0.11	-	1.423 ± 0.02	-	1.572 ± 0.31	-
0.5	1.311 ± 0.08	0.022 ± 0.007	1.199 ± 0.03	-	1.425 ± 0.01	-	1.386 ± 0.01	-
1	1.15 ± 0.1	-	0.632 ± 0.21	-	1.437 ± 0.01	-	1.443 ± 0.02	-
1.5	0.13 ± 0.06	-	0.202 ± 0.18	-	1.324 ± 0.15	-	1.398 ± 0.11	-
2	0.062 ± 0.06	-	0.677 ± 0.26	-	1.272 ± 0.1	-	1.25 ± 0.28	-
2.5	-	-	0.173 ± 0.15	-	0.731 ± 0.14	-	0.722 ± 0.16	-
3	-	-	0.143 ± 0.1	-	0.485 ± 0.39	-	0.304 ± 0.08	-
3.5	-	-	0.302 ± 0.2	-	0.539 ± 0.74	-	0.068 ± 0.08	-
4	-	-	-	-	0.194 ± 0.22	-	0.358 ± 0.55	-
4.5	-	0.010 ± 0	-	-	-	-	0.119 ± 0.03	-
5	-	-	-	-	-	-	-	-

Table (5.21) *Mean and Standard Deviation ± (n = 3) of the mean max OD_{600} values for* C. pleistocenium *cultured at 28°C under a range of pH values and salinity (% NaCl [wt/vol]) (n = 11).*

28°C	pH 7.5	pH 7.5	pH 7	pH 7	pH 6.5	pH 6.5	pH 6	pH 6
NaCl (%)	+	-	+	-	+	-	+	-
0	**1.357 ± 0.08**	0.262 ± 0.006	**1.432 ± 0.01**	-	**1.291 ± 0.06**	0.363 ± 0.1	**1.397 ± 0.07**	0.335 ± 0.04
0.5	**1.27 ± 0.08**	0.284 ± 0.002	**1.327 ± 0.02**	0.226 ± 0.002	1.152 ± 0.18	**1.232 ± 0.74**	**0.921 ± 0.37**	0.383 ± 0.11
1	**0.816 ± 0.4**	0.290 ± 0.005	**1.108 ± 0.07**	0.265 ± 0.012	**1.080 ± 0.13**	0.958 ± 0.9	**0.836 ± 0.06**	0.339 ± 0.05
1.5	**1.162 ± 0.12**	0.288 ± 0.007	**0.928 ± 0.14**	0.516 ± 0.22	**0.656 ± 0.54**	0.457 ± 0.06	0.464 ± 0.07	**0.609 ± 0.37**
2	**0.675 ± 0.4**	0.287 ± 0.007	**0.893 ± 0.12**	0.250 ± 0.007	**0.762 ± 0.51**	0.538 ± 0.08	**0.918 ± 0.44**	0.474 ± 0.03
2.5	-	0.308 ± 0.009	**0.539 ± 0.24**	0.262 ± 0.008	**0.746 ± 0.06**	0.477 ± 0.045	0.337 ± 0.04	**0.338 ± 0.03**
3	-	0.303 ± 0.003	**0.397 ± 0.14**	0.269 ± 0.014	0.372 ± 0.3	**0.499 ± 0.07**	0.129 ± 0.04	**0.445 ± 0.09**
3.5	-	0.326 ± 0.03	0.201 ± 0.13	**0.260 ± 0.003**	0.363 ± 0.38	**0.809 ± 0.48**	-	**0.383 ± 0.04**
4	-	0.374 ± 0.04	0.012 ± 0.001	**0.256 ± 0.03**	0.311 ± 0.06	**0.589 ± 0.02**	**0.501 ± 0.64**	0.425 ± 0.03
4.5	-	0.438 ± 0.26	0.015 ± 0.004	**0.163 ± 0.13**	0.066 ± 0.1	**0.566 ± 0.045**	-	**0.386 ± 0.02**
5	-	0.248 ± 0.007	-	**0.159 ± 0.21**	-	**0.514 ± 0.02**	-	**0.398 ± 0.12**

Table (5.22) *Mean and Standard Deviation ± (n = 3) of the mean max OD_{600} values for* C. pleistocenium *cultured at 30°C under a range of pH values and salinity (% NaCl [wt/vol]) (n = 11).*

30°C	pH 7.5	pH 7.5	pH 7	pH 7	pH 6.5	pH 6.5	pH 6	pH 6
NaCl (%)	+	-	+	-	+	-	+	-
0	**0.364 ± 0.6**	-	**0.61 ± 0.6**	-	**0.517 ± 0.59**	0.153 ± 0.09	**1.123 ± 0.16**	0.474 ± 0.33
0.5	**0.03 ± 0.007**	-	-	-	0.055 ± 0.035	**0.6 ± 0.25**	0.018 ± 0.01	**0.623 ± 0.4**
1	-	**0.013 ± 0**	-	-	0.145 ± 0.15	**0.914 ± 0.43**	-	**0.56 ± 0.1**
1.5	-	-	-	-	**0.851 ± 0.15**	0.264 ± 0.03	0.168 ± 0.25	**0.255 ± 0.02**
2	-	**0.012 ± 0.001**	-	-	0.076 ± 0.1	**0.808 ± 0.3**	0.654 ± 0.99	**0.96 ± 0.2**
2.5	-	**0.010 ± 0**	-	-	-	**0.425 ± 0.2**	-	**0.359 ± 0.1**
3	-	**0.012 ± 0.001**	-	-	-	**0.912 ± 0.34**	-	**0.135 ± 0.03**
3.5	-	-	-	-	-	**0.684 ± 0.64**	-	**0.376 ± 0.4**
4	-	-	-	-	-	**0.426 ± 0.16**	-	-
4.5	-	-	-	-	-	**0.583 ± 0.24**	-	-
5	-	-	-	-	-	-	-	**0.344 ± 0.26**

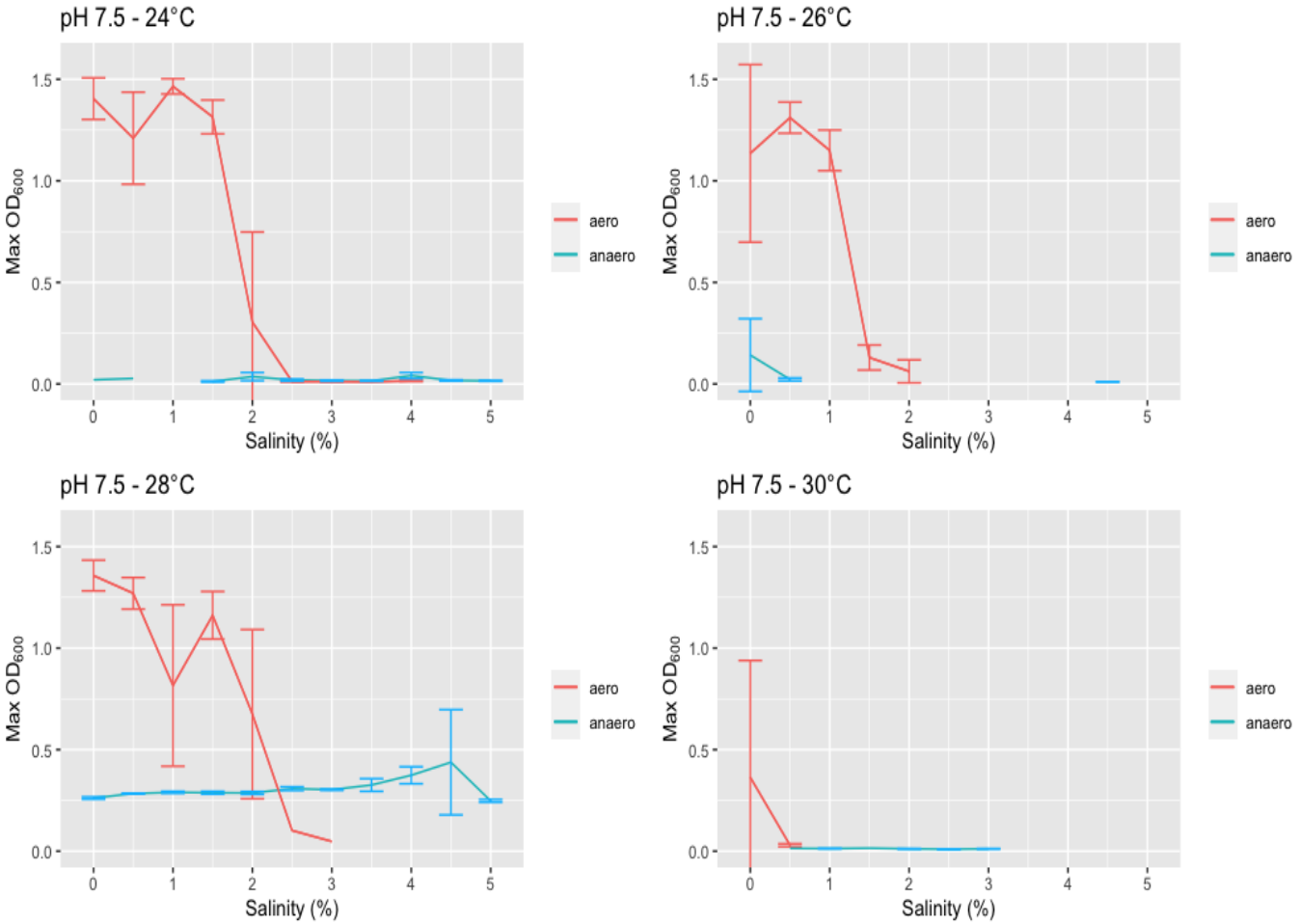

Figure (5.8) *Graph displaying mean maximal OD_{600} values obtained under pH 7.5 and a range of salinities (% NaCl [wt/vol] (n = 11), and temperatures for* C. pleistocenium. *Data presented as mean max OD_{600} values ± standard error of the means (SE) (n = 3).*

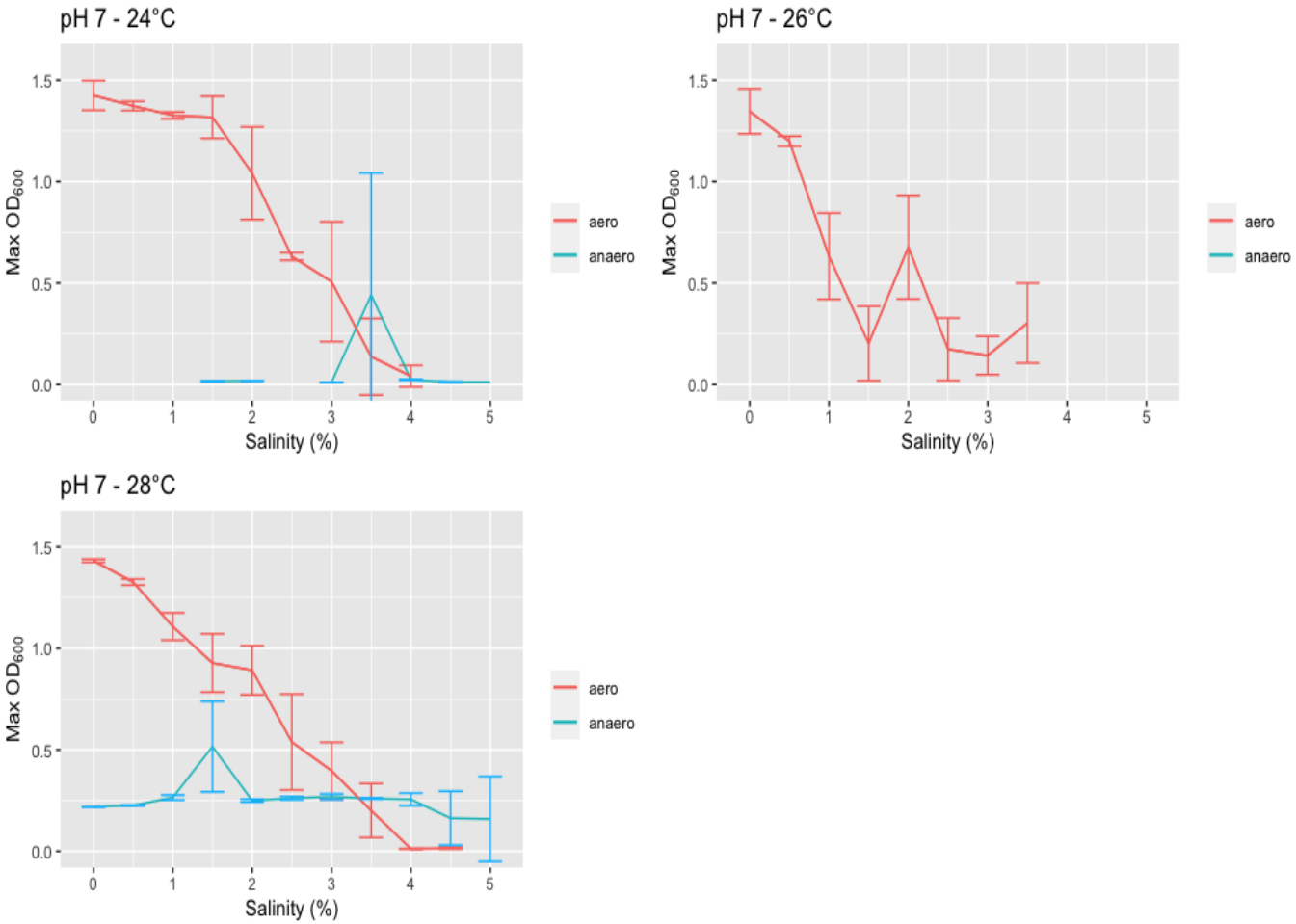

Figure (5.9) *Graph displaying mean maximal OD_{600} values obtained under pH 7 and a range of salinities (% NaCl [wt/vol] (n = 11), and temperatures for* C. pleistocenium. *Data presented as mean max OD_{600} values ± standard error of the means (SE) (n = 3).*

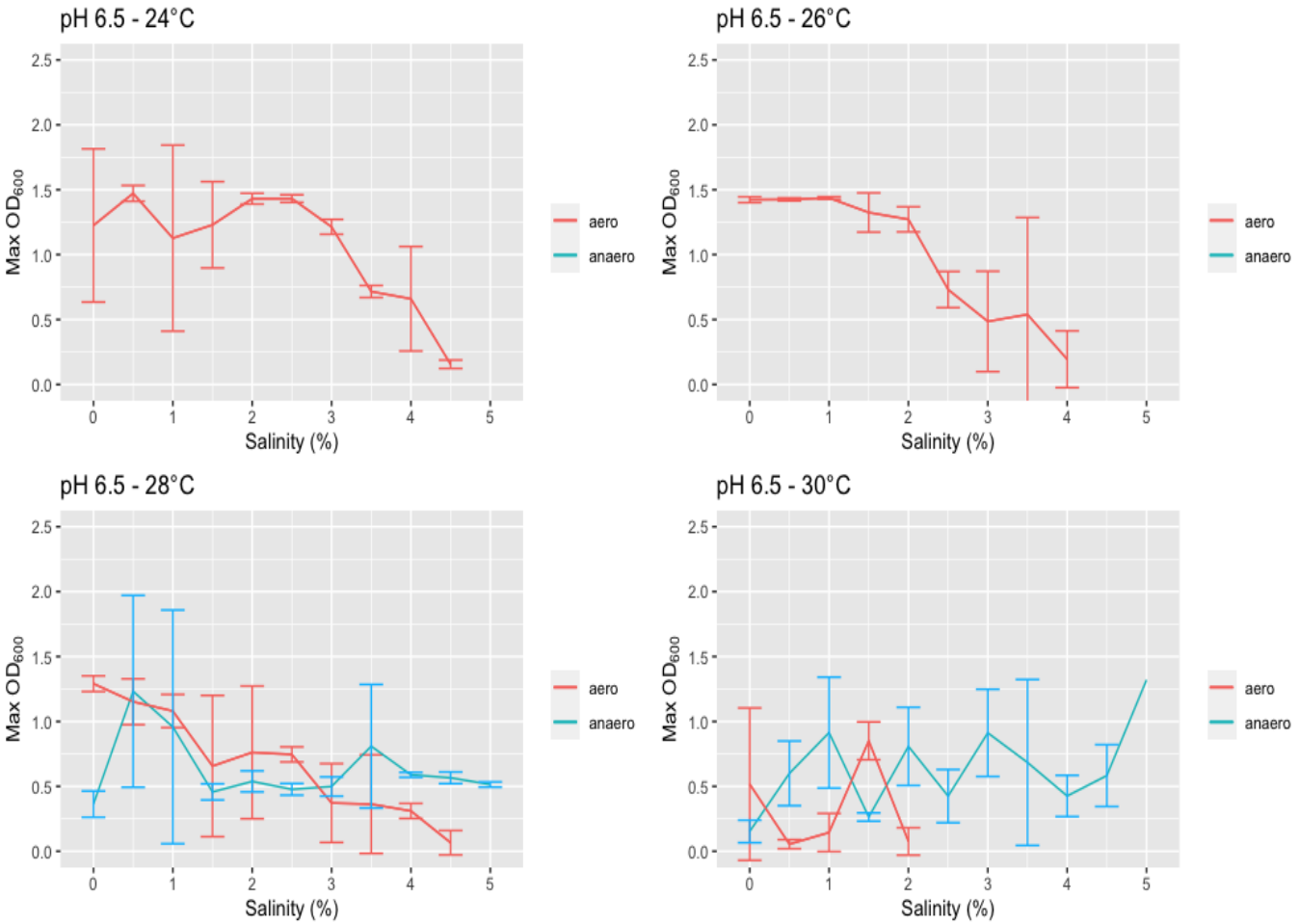

Figure (5.10) *Graph displaying mean maximal OD_{600} values obtained under pH 6.5 and a range of salinities (% NaCl [wt/vol] (n = 11), and temperatures for* C. pleistocenium. *Data presented as mean max OD_{600} values ± standard error of the means (SE) (n = 3).*

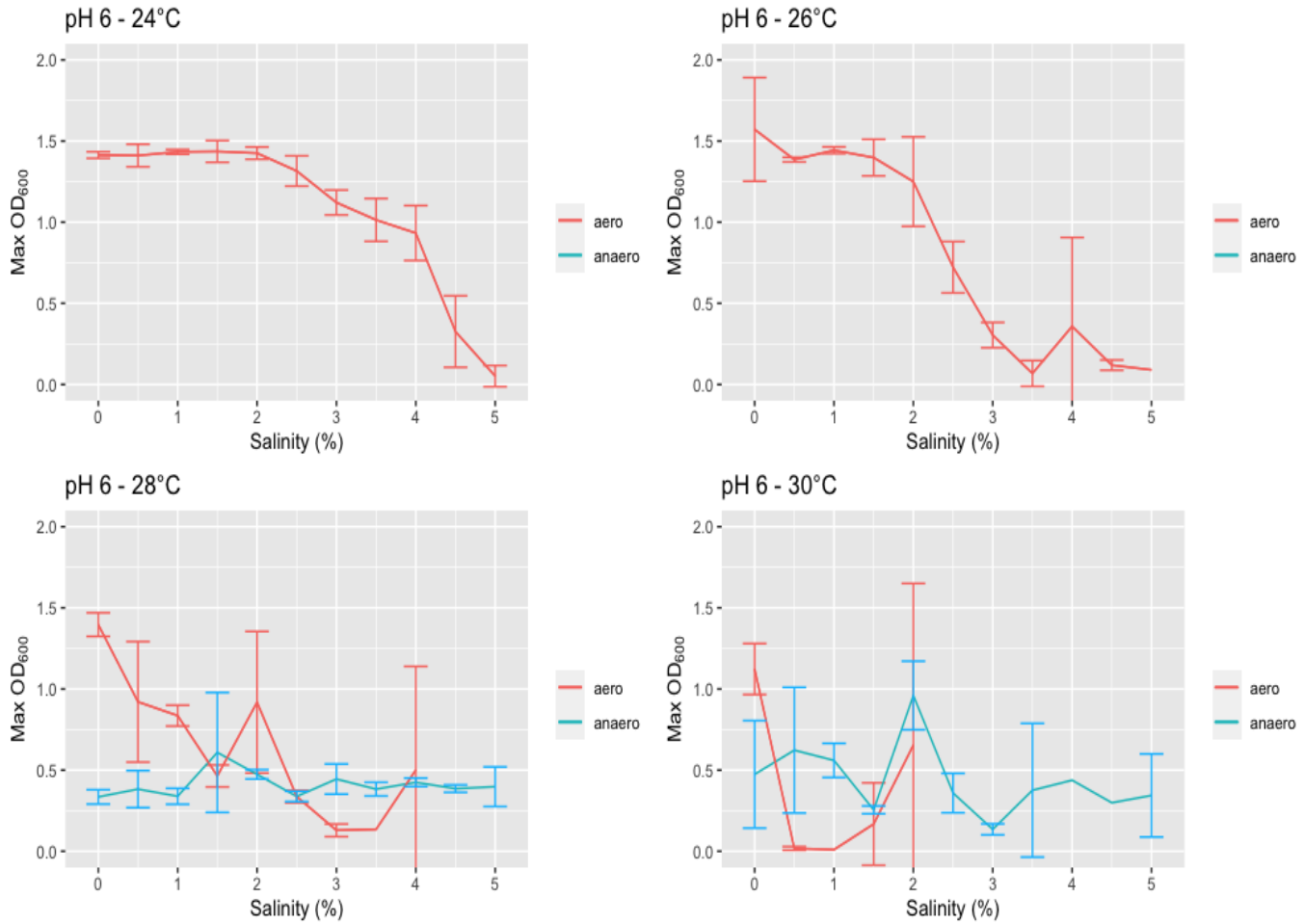

Figure (5.11) *Graph displaying mean maximal OD_{600} values obtained under pH 6 and a range of salinities (% NaCl [wt/vol] (n = 11), and temperatures for* C. pleistocenium. *Data presented as mean max OD_{600} values ± standard error of the means (SE) (n = 3).*

Table (5.23) *T-test results comparing aerobic and anaerobic maximal OD_{600} values obtained for* C. pleistocenium *cultured under a range of pH conditions and salinities (% NaCl [wt/vol]) (n = 11) at 24°C.*
Green indicates instances in which neither condition yielded measurable data, red indicates no aerobic growth and blue indicates no anaerobic growth.
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

24°C NaCl (%)	pH 7.5	pH 7	pH 6.5	pH 6
0				
0.5				
1				
1.5	$t(2) = 27, \mathbf{p < 0.01}$*	$t(2) = 21.7, \mathbf{p < 0.05}$*		
2	$t(2) = 1.05, p = 0.405$*	$t(2) = 7.77, \mathbf{p < 0.05}$*		
2.5	$t(3) = -2.64, p = 0.098$			
3	$t(4) = -2.97, p = 0.071$	$t(2) = 2.9, p = 0.167$*		
3.5		$t(3) = -0.88, p = 0.556$		
4	$t(4) = -2.93, p = 0.071$	$t(2) = 0.58, p = 0.618$*		
4.5				
5				

Table (5.24) *T-test results comparing aerobic and anaerobic maximal OD_{600} values obtained for* C. pleistocenium *cultured under a range of pH conditions and salinities (% NaCl [wt/vol]) (n = 11) at 26°C.*
Green indicates instances in which neither condition yielded measurable data, red indicates no aerobic growth and blue indicates no anaerobic growth.
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

26°C NaCl (%)	pH 7.5	pH 7	pH 6.5	pH 6
0	$t(3) = 2.92, p = 0.061$			
0.5	$t(3) = 22.4, \mathbf{p < 0.001}$			
1				
1.5				
2				
2.5				
3				
3.5				
4				
4.5				
5				

Table (5.25) *T-test results comparing aerobic and anaerobic maximal OD_{600} values obtained for* C. pleistocenium *cultured under a range of pH conditions and salinities (% NaCl [wt/vol]) (n = 11) at 28°C.*
Green indicates instances in which neither condition yielded measurable data, red indicates no aerobic growth and blue indicates no anaerobic growth.
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

28°C				
NaCl (%)	**pH 7.5**	**pH 7**	**pH 6.5**	**pH 6**
0	$t(2) = 24.9, \mathbf{p < 0.01}$*	$t(3) = 204.9, \mathbf{p < 0.001}$	$t(4) = 13.7, \mathbf{p < 0.01}$	$t(4) = 21.7, \mathbf{p < 0.001}$
0.5	$t(2) = 22, \mathbf{p < 0.01}$*	$t(2) = 128.2, \mathbf{p < 0.001}$*	$t(4) = -0.18, p = 0.864$	$t(4) = 2.7, p = 0.106$
1	$t(2) = 2.3, p = 0.186$*	$t(4) = 21.3, \mathbf{p < 0.001}$	$t(2) = 0.23, p = 0.86$*	$t(3) = 10, \mathbf{p < 0.01}$
1.5	$t(1) = 10.5, p = 0.099$*	$t(4) = 2.69, p = 0.091$	$t(2) = 0.63, p = 0.739$*	$t(4) = -0.7, p = 0.718$
2	$t(2) = 1.61, p = 0.248$*	$t(2) = 9.18, \mathbf{p < 0.05}$*	$t(4) = 0.7, p = 0.739$	$t(2) = 1.8, p = 0.350$*
2.5		$t(2) = 2, p = 0.240$*	$t(4) = 6.3, \mathbf{p < 0.01}$	$t(4) = -0.03, p = 0.976$
3		$t(2) = 1.58, p = 0.279$*	$t(4) = -0.7, p = 0.79$	$t(4) = -5.4, \mathbf{p < 0.05}$
3.5		$t(2) = -0.76, p = 0.524$*	$t(3) = -1.1, p = 0.707$	
4		$t(2) = -13.66, \mathbf{p < 0.05}$*	$t(3) = -8.3, \mathbf{p < 0.01}$	$t(1) = 0.17, p = 0.975$*
4.5		$t(2) = -1.93, p = 0.240$*	$t(4) = -0.6, \mathbf{p < 0.01}$	
5				

Table (5.26) *T-test results comparing aerobic and anaerobic maximal OD_{600} values obtained for* C. pleistocenium *cultured under a range of pH conditions and salinities (% NaCl [wt/vol]) (n = 11) at 30°C.*
Green indicates instances in which neither condition yielded measurable data, red indicates no aerobic growth and blue indicates no anaerobic growth.
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

30°C NaCl (%)	pH 7.5	pH 7	pH 6.5	pH 6
0			$t(2) = 1.1, p = 0.395$*	$t(3) = 2.5, p = 0.278$
0.5			$t(2) = -3.7, p = 0.100$*	$t(2) = -2.4, p = 0.278$*
1			$t(3) = -2.4, p = 0.125$	
1.5			$t(4) = 6.8, \mathbf{p < 0.05}$	$t(2) = -0.6, p = 0.613$*
2			$t(4) = -3.98, \mathbf{p < 0.05}$	$t(4) = -0.5, p = 0.631$
2.5				
3				
3.5				
4				
4.5				
5				

5.5 Discussion

The availability of metabolic energy may be the most fundamental property governing environmental habitability and ecosystem structure [Mccollom and Amend, 2005; Hoehler 2007]. Understanding how the availability of energy might change the capacity of life to propagate in multiple extreme environments is essential when determining the potential habitability window for life.

One of the most profound changes in the history of life on Earth is the transition of its atmosphere from anoxic to oxic [Kopp et al. 2004; Sesssions et al. 2009]. This introduction of atmospheric oxygen happened around 2.4 billion years ago and then again approximately 700 million years ago, allowing for the development of complex multicellular life over a large expanse of time (Catling et al., 2005). Much is theorised about how the introduction of oxygen into Earths atmosphere brought about the conditions suitable for complex life to evolve [Catling et al. 2005(2); Buick et al. 2008], yet our understanding of how the addition of a newly exploitable chemical energy source may have altered the limits of microbial life under extreme conditions is insubstantial.

It is known that ATP yields differ considerably between aerobic and anaerobic respiration [Unden and Bongaerts 1997; King 2005] and the presence of environmental factors such as pH, NaCl (%) and temperature can significantly alter the efficacy of transmembrane ion-gradients [Konings et al. 1994; Lane and Martin 2012], yet few laboratory studies have been done to systematically explore the interactions of three or more stresses on the limits of microbial growth under both aerobic and anaerobic conditions. Harrison et al. 2015 demonstrate in a report assessing the growth ranges of 241 prokaryotic strains that anaerobically respiring microorganisms display a narrower range for cell division than aerobic strains, but the lack of information on how the theoretical energy yields of these metabolic processes differs under the combined influence of multiple extremes for the same organism are not represented in these data. To provide a better understanding of the limits to microbial growth employing different modes of metabolism, these maps should be constructed using microbial growth data in which multiple extremes are experienced in combination using facultative anaerobic strains cultured under both aerobic and anaerobic conditions. This study contributes to this paucity of information. Though there is significant data demonstrating different growth rates between aerobic and anaerobic strains, this research addresses a lack of comparative data by using the same organism under

combinations of environmental stresses to assess the differential growth response under standard and theoretically energy limiting conditions.

This study aims to quantify the combined effect of stresses of salinity, pH and temperature on three facultative anaerobic organisms (*Halomonas hydrothermalis*, *Escherichia coli* and *Carnobacterium pleistocenium*) under aerobic and anaerobic culture conditions. These model organisms were selected due to their ability to propagate under a broad range of these stress parameters both aerobically and anaerobically [Kaye and Barros 1, 2004: Unden and Bogaerts. 1997], and in the case of *C. pleistocenium* because the range of growth parameters is much narrower than *H. hydrothermalis* and *E. coli* [Pikuta et al. 2005], thus allowing for an increased understanding of the effect of multiple extremes over of more confined parameter space. The experimental parameters of salinity, pH and temperature were selected due to their established limits to life and prevalence in natural environments [Schleper et al. 1996; Suzuki et al. 2014; Blum et al. 2009].

The work presented in this chapter demonstrates that when cultivated under aerobic and anaerobic conditions, the facultative anaerobic strains *H. hydrothermalis*, *E. coli* and *C. pleistocenium* display significantly different aerobic and anaerobic growth when cultured under a combination of stresses of pH, NaCl concentration [NaCl wt/vol] and temperature.

These data show the model strain *H. hydrothermalis* grew better under aerobic culture conditions than anaerobic culture conditions, however, conflicting with the hypothesis that aerobic respiration might allow for a broader range of cell division under extreme conditions, *H. hydrothermalis* displays increased growth yields at mildly acidic pH under optimal and moderately supra-optimal temperatures under anaerobic conditions. The increased growth under NaCl (%) and acidic pH and anaerobic conditions shifts to neutral pH conditions with increased temperatures. Consistent with the hypothesis that aerobic respiration would provide more energy, aerobic *H. hydrothermalis* cultures grew better at supra-optimal temperatures and pH 6.

Similar results were obtained with *E. coli* cultures under aerobic and anaerobic conditions. The results show that when cultured aerobically, *E. coli* grew better under low saline conditions, however, when cultured anaerobically, cultures displayed increased growth under high NaCl concentrations. Additionally, anaerobic cultures of *E. coli* exhibited increased growth under acidic

culture conditions particularly under high salinities. These results are displayed throughout the temperature range tested for this strain.

Growth data collected for *C. pleistocenium* reveals a similar pattern. Aerobic cultures grew best under optimal and moderately supra-optimal temperatures for all pH values and salinities, however anaerobic cultures grew better under optimal temperatures at pH 7.5 and high salinity. Additionally, when cultured under supra-optimal temperatures, anaerobic cultures grew better under the range of pH values and under increased salinities.

The strains employed in this chapter displayed differences in growth yields over a range of salinities, pH values and temperatures between aerobic and anaerobic growth conditions. Contrary to knowledge that aerobic and anaerobic energy yields can vary, and anaerobic organisms have been shown to display a narrower range of tolerance towards the extremes of this study individually (Unden and Bongaerts, 1997; King, 2005: Harrison et al. 2015), these results demonstrate the complex interactions of multiple environmental extremes of NaCl (%), pH and temperature covering optimal, sub- and supra-optimal variations, have distinctly different effects when organisms are exposed to aerobic and anaerobic environments.

Two of the model strains used in this study (*H. hydrothermalis* and *E. coli*) displayed increased growth under acidic conditions when cultured anaerobically when compared with aerobic growth under the same conditions. Additionally, this study demonstrates that the limits of life can be extended both under aerobic and anaerobic conditions, with previously established limits to pH and temperature for two of the model organisms (*H. hydrothermalis* and *C. pleistocenium*) (see section 3.2 and Chapter 4) extended under both aerobic and anaerobic conditions and a combination of pH and NaCl concentration.

These data have significant implications when considering the affect the rise of oxygen during The Great Oxidation Event may have had on Earths anaerobic microbial population. Our current understanding about the respective energy yields from aerobic and anaerobic respiration suggests the introduction of atmospheric oxygen may have provided enough energy to allow life to extend the boundaries of habitability, indeed this newly available energy source allowed for the development of complex multicellular life [Blankership and Hartman 1998; Buick 2008; Catling et al. 2005(2)]. However, cellular metabolism is known to be affected by a range of environmental variables including pH, NaCl concentration

and temperature [Jin and Kirk 2018] and the energy requirements to deal with these extremes in combination are complex. For example, maintaining a pH gradient across the cellular membrane requires available energy which may be significantly impacted by additional environmental factors, such as temperature, salinity or pressure, that in turn are themselves affected by the multiple factors impacting cellular energetics [Padan et al. 2001; Jin 2012; Jin and Bethke 2002; Hoehler 2007].

The data collected in this chapter suggest energetic limitations have significantly different impacts when experienced in aerobic or anaerobic environments for facultative anaerobic organisms. The complex balance of biochemical impacts imposed on *H. hydrothermalis*, *E. coli* and *C. pleistocenium* by stresses of NaCl (%), pH and temperature are shown to prove detrimental at certain intersects controlled by particular combinations of these parameters. For example, electrochemical proton potential and cellular ATP yields for *E. coli* under aerobic and anaerobic conditions have previously been shown to be similar [Tran and unden, 1998], however, the data from this study suggest that an organisms capacity to cope with extremes of salinity vary with pH and in turn are controlled by environmental temperature which has significantly different effects under aerobic and anaerobic conditions.

The results suggest that the rise in atmospheric oxygen through The Great Oxidation Event may have released some organisms from the constraints of anaerobic respiration and extended the limits of life under certain environmental conditions. However, under some conditions such as low pH, high temperature and saline conditions, such as those found in deep-sea hydrothermal habitats or continental hot springs [Prokofeva et al. 2005], microbes may have found little or no improvement from the introduction of this newly available energy source. These data further demonstrate that it is essential to assess the habitability of natural environments with a deeper understanding of the interplay between concomitant physicochemical parameters, with a particular focus on cellular energetic demands under the impact of multiple extremes.

5.5.1 Limitations

Though the experimental work presented in this chapter allowed for the comparison between aerobic and anaerobic growth under multiple extremes using facultative anaerobic strains, there are limitations to take into consideration.

Natural environments are subject to a multitude of conditions that are not explored in this study, such as UV radiation, desiccation and heavy metals. To gain a better understanding of how the effects of multiple extremes differ under oxic and anoxic conditions, one should consider exploring the interactions between additional extremes that are more commonly found in anoxic environments such as high pressure. Despite this limitation, it is clear from these data that in oxic and anoxic environments multiple extreme parameters interact to significantly change the limits of life.

5.5.2 Future Work

The data reported in this chapter focuses on the response of three facultative anaerobic strains to multiple extremes under oxic and anoxic conditions. Similarly to the future work proposed in chapter 4, further experimental work using a diverse selection of mesophilic, extremophilic and extremotolerant facultative anaerobes would provide additional data on the impact of multiple extremes in the presence and absence of oxygen, and should be conducted using a variety of extreme conditions. A new top of investigation could include the growth response or faculatative anaerobes to a combination of previously untested stresses (eg, heavy metals, UV radation, perchlorate salts), and a detailed examination of the physiological response within cellular membranes under these conditions. When considering the prevalence of microbes in the deep-subsurface and the knowledge that much of the high-pressure deep-subsurface environment is anoxic [Teske 2005], further work exploring how the additional physical stress of pressure may further define the habitability of these natural habitats would prove beneficial, particularly in light of the data reported in Chapter 6.

5.5.3 Conclusions

For an organism to propagate in a given environment requires energy at a sufficient rate to meet the energetic demands, and the potential for meeting this demand under extreme conditions further defines the habitability of an environment. How an increase in newly available energy sources, such as atmospheric oxygen during The Great Oxidation Event, may change the limits of life under multiple extremes is unknown. Contrary to our understanding that anaerobic respiration produces lower ATP yields than aerobic respiration, the

work presented in this chapter demonstrates that when stresses of NaCl(%), temperature and pH are experienced in combination, they act to limit microbial growth in significantly different ways under oxic and anoxic conditions. These findings further highlight the need for a better definition of the limits of life in extreme conditions and a more accurate characterisation of the boundaries of habitability with a particular emphasis on cellular energetics.

Chapter 6

Under pressure: the synergistic effect of simulated hydrostatic pressure on the limitis of life under multiple stresses

6.1 Introduction

It is important to take into consideration the natural environment of an organism when determining both what constitutes an extreme environmental parameter, and how best to examine the effect of those conditions on microbial survival and propagation. These data further demonstrate the multiplicative effect of environmental stresses on microbial propagation and viability, further outlining the limits of life under a combination of multiple extreme parameters.

Although we usually think of high pressure environments as extreme, much of life on Earth is at high pressure in the deep ocean and subsurface. From that perspective, life at one atmosphere might be seen to be low pressure adapted. With this in mind, when attempting to determine the effects of the additional physical parameter of simulated hydrostatic pressure, one must consider that in some instances higher than atmospheric pressure should not be regarded as an extreme condition. Instead, in instances such as this study, atmospheric pressure can be viewed as the stress parameter. This is due the model organism

(*Halomonas hydrothermalis*) having been isolated from a natural environment that experiences higher than atmospheric pressure; a deep-sea hydrothermal vent system at approximately 2580 metres depth in Pacific Ocean (Kaye et al., 2004).

This chapter explores the effect of simulated hydrostatic pressure on microbial propagation using the same model organism as employed in chapter 4 (*H. hydrothermalis*), due its ability to tolerate a wide range of growth conditions (see methods for details). The aim of the experiment described in this chapter is to provide a further understanding of the effect of multiple extremes on microbial propagation under the influence of a proposed physical environmental stress, and whether such an environmental parameter could, or should, indeed be considered a stress to the selected model organism. Further to this, we aim to provide a better understanding of the boundaries of habitability in addition to the work already completed examining the effects of the combination of supra-optimal temperatures, acidic pH and variations in salinity on the growth of *Halomonas hydrothermalis* in Chapter 4.

For the experimental work conducted in this chapter is was necessary to develop and assemble new apparatus in collaboration with The Centre for Science at Extreme Conditions (CSEC), The University of Edinburgh. The high-pressure vessel obtained from Parr Instrument Company, Moline, IL, USA, is capable of achieving pressures up to 200 bar and required the manufacture of a safety cage and a specially designed insert capable of holding the syringes used for this study (see figures 6.1 and 6.2 for details). Due to its biological nature, it was essential that the area within which the research was carried out was classified as a category I laboratory and health and safety measures were adhered to accordingly. This collaborative effort has set in place the foundations for future students and researchers to continue work on the effects of simulated hydrostatic pressure extremes on microbes and deepened the cooperative relationship between The School of Physics and Astronomy and CSEC within The University of Edinburgh.

In this chapter, data collected under simulated hydrostatic pressure was directly compared with results collected from experimental work performed using the same setup procedure but conducted outside the pressure vessel under atmospheric pressure conditions. There is limited research assessing the effect of multiple naturally occurring environmental stresses found in high pressure environments, in particular those examining the effect salinity and pH under simulated hydrostatic pressures. With this in mind, this study aims to address this gap in our understanding of the effect of concomitant extremes under

the physical constraint of high pressures to test the hypothesis that increased simulated hydrostatic pressure will extend the model organisms capacity to deal with variations in NaCl concentration and environmental pH. The results from this chapter further illustrate the importance of taking into consideration the natural habitat of the organism when raising questions of the habitability of a particular environment. The work presented in this chapter has been submitted for publication in Astrobiology.

6.2 Background

6.2.1 Life under pressure

Pressure is a fundamental parameter of Earths biosphere that has played a key role in the evolution and distribution of life on Earth, and it is theorised that the origin of life may have occurred under the influence of high pressure (Nisbet and Sleep, 2001). The deep-sea piezosphere (>1000 m depth and >100 bar pressure (Bartlett, 1999) accounts for approximately 65 to 75% of the total ocean and is believed to host approximately 62% of Earths biosphere (Cario et al., 2019; Fang and Bazylinski, 2008), though it has previously been proposed to host as much as 88% (Jannasch and Taylor., 1984). The subsurface (defined as terrestrial habitats below 8 m and marine sediment below 10 cm) is predicted to be a major habitat for prokaryotes that exceed numbers found in other components of the biosphere (Whitman et al., 1998). Other high-pressure environments include deep lakes and subglacial lakes (Bartlett, 2002; Pikuta et al., 2007), continental and oceanic crusts, hydrothermal vents and cold seep systems (Jones et al., 2018), and these sites are thought to share some physical and chemical similarities with extra-terrestrial high-pressure environments such as the interiors of Enceladus and Europa (Vance et al., 2016), and the Martian subsurface (Stevens et al., 2015). It is evident that an abundance of life on Earth exists under the influence of higher than atmospheric pressures, and the effect of these pressures imposed on organisms in addition to other environmental factors such as salinity and pH are yet to be fully understood. The necessary adaptations to deal with these extremes individually are well established (Baker-Austin and Dopson, 2007; Konings et al., 2002; Krulwich et al., 2011; Oren, 2006), yet the potential synergistic or antagonistic nature of a combination of these stresses in high pressure environments requires a more robust understanding of the physical

constraints imposed on life under high pressures conditions.

6.2.2 Pressure and other environmental factors

Earths subsurface habitats, and the microbial life that occupy them, in addition to being subject to higher pressures when compared with surface/above surface conditions, often experience variations in other environmental extremes such as extremes in pH and temperature (Jones et al., 2018). For example, hydrothermal vent systems experience pressures exceeding 300 bar in addition to temperature variations ranging from 2°C to more than 400°C (Pledger et al., 1994), and hydrothermal vent fluid measurements from the East-Pacific ridge and the Stevenson Island vents in Yellowstone Lake are shown to be slightly acidic (pH 5.1 5.4 and 4.2 4.5 respectively) (Ding et al., 2005; Tan et al., 2017). High hydrostatic pressures have also been shown to increase minimal and maximal growth ranges in hyperthermophiles from hydrothermal vent systems (Canganella et al., 1997; Holden and Baross, 1995; Pledger et al., 1994).

Increased pressure causes changes in bacterial membrane fluidity through alterations in lipid volume and packaging which restricts permeability and disrupts protein-lipid interactions within the membrane (Brooks, 2014; Winter and Jeworrek, 2009). Additionally, high pressure is known to disrupt crucial cellular processes such as essential DNA processes (replication, translation and transcription) (Macgregor, 2002), metabolic activity, protein function and synthesis (Northrop, 2002), and enzyme activity (Abe, 2007; Huang et al., 2016). Microbial adaptations to pressure conditions and the effect on biological function are not discussed in detail here but are found in Abe (2007), Mota et al. (2013),Huang et al. (2016) and Winter and Jeworrek (2009). It is clear the potential antagonistic effect pressure might have on microorganisms depends on other physicochemical parameters having influence, for example *Escherichia coli* has been shown to display decreased pressure tolerance when cultivated under a combination of acidic pH and low temperature (Bartlett, 2002). Moreover, when other extremes effect organisms in addition to high pressures, such as low temperatures, they can display synergistic effects on microbial survival due the overlapping mechanisms of adaptation to these extremes (Winter and Jeworrek, 2009). High hydrostatic pressures have also been shown to increase minimal and maximal growth ranges in hyperthermophiles from hydrothermal vent systems (Canganella et al., 1997; Holden and Baross, 1995; Pledger et al., 1994). Indeed,

the classification of piezophiles (pressure loving organisms; see 2.3.4. for further details) have been proposed to rely on both temperature and pressure maximums for growth due to the overlapping nature of their influence on high-pressure deep-sea habitats (Fang et al., 2010). In addition, increased hydrostatic pressure does not change the pressure differential through the membrane, but increased osmotic pressure outside the cell results in an alteration of turgor pressure (Jebbar et al., 2015; Martin et al., 2002). Understanding the interplay between multiple stresses under physical constraints provides further insight into characterising the net effect of multifarious environmental extremes on microbial viability. Exploring the impact of the combined effect of salinity and pH under simulated hydrostatic pressures is the primary focus of the experimental work presented in this chapter.

6.3 Methods and materials

6.3.1 Experimental work

Selection of model organisms

Halomonas hydrothermalis DSM 15725 was secured from the German collection of Microorganisms (DSMZ, Braunschweig, Germany). *H. hydrothermalis* exhibits cellular division at NaCl concentrations between 0.5% and 22% [wt/vol](optimal range of 4% to 7% [wt/vol]), pH between 5 and 12 (optimal range of 7 to 8) and temperatures between 2°C and 40°C (optimal growth at 30°C) (Kaye et al., 2004). Additional strain information can be found in the General Methodology section 3.2.

Microbial preparation

Starter cultures were obtained by culturing of the bacterium in minimal marine media (MMM) (Östling et al., 1991), altering the level of glucose to produce an MMM with 0.5% glucose composition, with 1.63% NaCl [wt/vol] (pH 8). Agar plates were prepared using MMM with the addition of Agar Bacteriological No. 1 at a concentration of 1.5% wt/vol. *H. hydrothermalis* starter cultures were prepared by transferring cells from agar plates to ∼30 mL MMM broth in a 250 mL conical flask with a foam bung. The culture was grown for 24 hours in a

shaking incubator (30°C, 120 rpm).

Growth assays

Growth of *H. hydrothermalis* was examined in Minimal Marine Media at a variation of NaCl concentrations (0.95%, 1.41%, 1.87%, 2.33%, 2.79%, 3.25%, 3.72%, 4.18%, 4.64%, 5.10%, 5.56%, 6.03%, 6.49%, 6.95%, 7.41%, 7.87%, 8.34%, 8.80%, 9.14%), pH values (8, 7 and 6) and under simulated hydrostatic pressures (50 and 150-bar) within a high pressure vessel (Parr Instrument Company, Moline, IL, USA). Starter culture (1 mL) was extracted from the conical flask and diluted with fresh MMM to a final cell density equivalent to an optical density at 600 nm (OD_{600}) of 0.2 within a 15 mL tube (Sarstedt, Nmbrecht, Germany).

NaCl solutions were prepared in individual 2 mL Eppendorf tubes to a total volume of 1480 μL, after which 20 μL of starter culture was added. Eppendorf cultures were loaded into a plastic syringe (BD Plastipak, Thermo-Fisher Scientific, Bishop Meadow Road, Loughborough, UK) up to a volume of 600 μL, heat sealed and placed within the pressure vessel insert (Figure 6.2). For each salinity, additional syringes were prepared using 600 μL from the same Eppendorf cultures and placed on a wire rack. The remaining solution was plunged out of the syringe into a cuvette for OD_{600} starter readings using a DR 5000 UV-Vis Spectrophotometer (Hach Company, Dsseldorf, Germany). A maximum of 12 samples were loaded into the pressure vessel via the pressure vessel insert specifically designed and manufactured for this study (Figure 6.1). The insert was made from Aluminium alloy 5052 due to its corrosive resistance and ability to cope with industrial atmospheres, and sand blasted to ensure a surface appropriate for disinfection in the event of a syringe rupture.

A total of 4 replicates per saline condition were prepared to account for the occurrence of a syringe rupture under pressurisation, in which case the required number of statistically necessary samples (i.e., 3) were more likely to remain intact. Upon loading, one sample was removed at random and the thermometer occupied the space of the removed sample and the vessel temperature was recorded using the FLUKE 52 II Thermometer.

After sample preparation and loading, the vessel was pressurised with nitrogen gas through a Swagelok NG-4 to the desired pressure and stored behind a ballistic shield for the 24-hour experiment period. During gas loading,

temperature readings were monitored to ensure internal vessel temperatures did not exceed 35°C. To maintain the internal vessel temperature at the desired 30°C, the vessel was partly (approx. 2/3) submerged in a water bath (Grant Instruments, Cambridge, UK) at 33°C which is held in place with specially designed clasps (Figure 6.2a) and maintained an internal vessel temperature of approximately 30°C. During all periods at which the vessel was pressurised it remained within a specially designed safety cage (Figure 6.2b) behind the ballistic shield. Upon completion of the 24-hour experimental period the gas was released through a 0.22 μm Millipore filter attached to the vessel via a Braun filter holder attached by plastic hosing and cable ties. The vessel remained behind the safety of the ballistic shield during de-pressurisation. Samples stored on the wire rack for growth at atmospheric pressure were placed in a warm room at 30°C for the duration of the 24-hour experimental period. Following this, high pressure and atmospheric pressure samples were plunged into individual cuvettes and OD_{600} measurements taken to provide a measurement of cell density after a 24-hour period for comparison.

Data analysis

The linear relationship between NaCl (%) and mean OD_{600} values were assessed using Pearsons product-moment correlation. Significant differences in measured OD_{600} values were assessed using a two-sample independent Students t-test. To assess the equality of variance between samples a Bartletts test was performed, for any samples displaying unequal variance a Welchs t-test was performed (marked with an asterisk in the results tables). The Benjamini-Hochberg Procedure was carried out on resulting p-values to decrease the false recovery rate. Bartletts test, Students t-test, Welchs t-test, Pearsons product-moment correlation and images were performed and created using RStudio v1.1.453 (RStudioTeam, 2018) with the package's 'car' and 'ggplot2'.

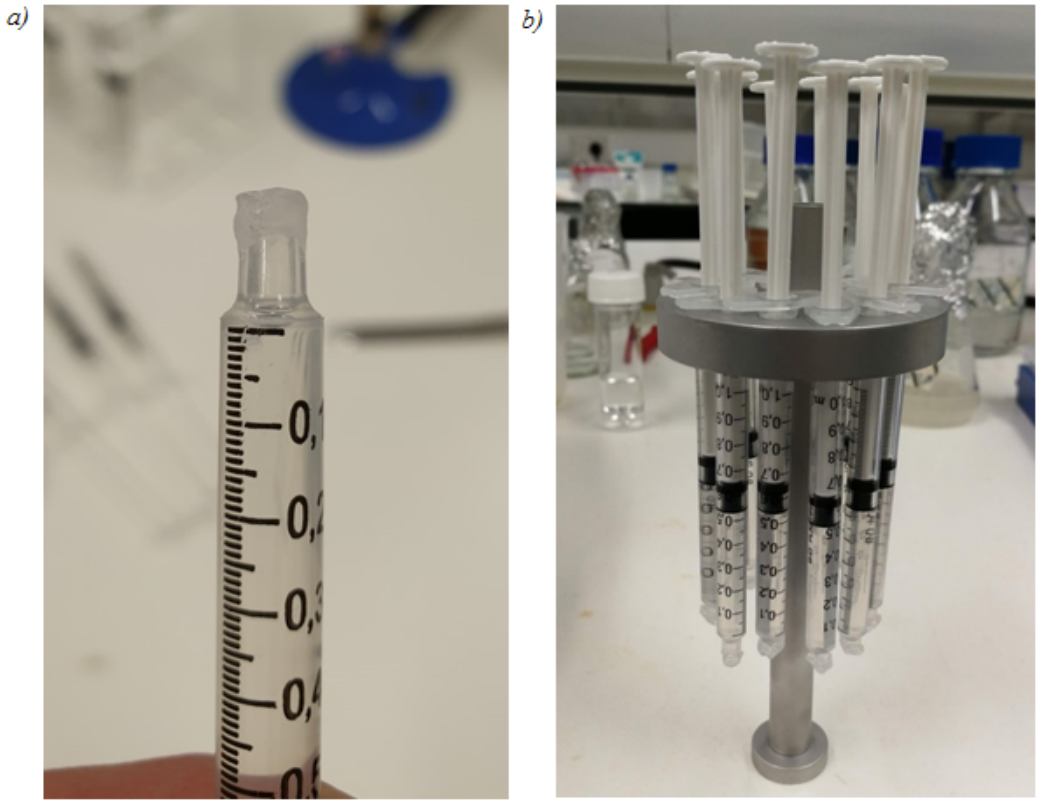

Figure (6.1) *a) Heat-sealed plastic syringe loaded with a culture sample in minimal marine media. b) Pressure vessel insert, designed for this study, holding heat-sealed samples ready for pressurisation. The insert was made from Aluminium allow 5052 due to its corrosive resistance and ability to cope with industrial atmospheres.*

Figure (6.2) *a) Pressure vessel setup. Pressure vessel is loaded into the safety cage (b) and sits in a water bath held in place with specially designed metal clasps, set to maintain the internal temperature of the vessel at 30° C. A Swagelok NG-4 hose is used to pressurize the vessel using nitrogen gas and the internal temperature of the vessel is measured using a FLUKE 52 II Thermometer. Upon gas release, the ejecting gas passes through a 0.22 µm Millipore filter attached to the vessel via a Braun filter holder attached by plastic hosing and cable ties to ensure, in the event of a syringe rupture, a sterile environment is maintained. b) Safety cage designed to house and support the pressure vessel during the experimental period.*

6.3.2 Preliminary work

Preliminary tests were performed to assess both the efficacy of the pressure vessel and the experimental setup:
Heat-sealing of the plastic syringes were performed using a number of methods to determine the most appropriate and efficient method for sealing. Methods were as follows:

- leaving the tip of the syringe free of liquid, it was exposed to the flame of a Bunsen burner for several seconds (being sure not to produce any smoke) and then the soft syringe tip was rolled on a sterilised metal spatula to seal the opening.
- leaving the tip of the syringe free of liquid, sterilised metal pliers were heated and used to clamp the syringe tip closed.

Both methods were tested within the pressure vessel under 150 bar to ensure they remain sealed at high pressure. The method of sealing via Bunsen burner and rolling on a metal spatula was shown to be the most efficient and effective method, and therefore the method carried forward for this experimental work. Further preliminary test runs were carried out with heat-sealed plastic syringes containing only deionized water ($n = 5$) and Minimal Marine Media ($n = 5$). The aim of this test run was to ensure appropriate experimental setup (i.e. heat-sealed syringes function correctly) and to assess the effect of pressure on the media used in the study (e.g. expulsion of gases). These test runs were conducted at 50-bar for a period of 1 hour, after which the vessel was de-pressurised, the syringes checked for ruptures and the media assessed for potential expulsion of gases. Once completed and proven successful at 50-bar, tests were repeated at 150-bar for 24 hours and the checks repeated.

Preliminary runs were performed with an empty pressure vessel to ensure appropriate maintenance of desired pressure for the duration of the experiment (24 hours). A test run was performed at 50-bar over a 1-hour period to identify any potential gas leak from the vessel, hosing or regulators at lower pressure. Internal pressure was recorded every 10 minutes for the first hour, then every hour for the following 5 hours of each test period (Figure 6.3a). Upon successful completion, additional test runs were performed at 100 bar (Figure 6.3b) and 150 bar (Figure 6.3c) to assess the vessel for gas leakage under higher

pressures over a 6-hour period, with internal pressure again recorded every 10 minutes for the first hour, then every hour for the following 5 hours of each test period.

Following completion of the aforementioned preliminary runs, a system check for gas release was performed. This consisted of assessing the speed of gas released upon a minimal turn of the gas release valve. If, after only a small turn, the release of gas was at a safe and slow level then the valve could be turned an additional quarter turn. This process was repeated until a quick but safe level of gas was released from the vessel, noting the final number of quarter-turns for future experimental runs. This was to ensure that upon completion of a run the release of gas is not conducted in a manner that results in an immediate release of all pressurised gas from within the chamber, which is a potential safety risk and may nullify the experiment through excessive expulsion of the syringe plunger.

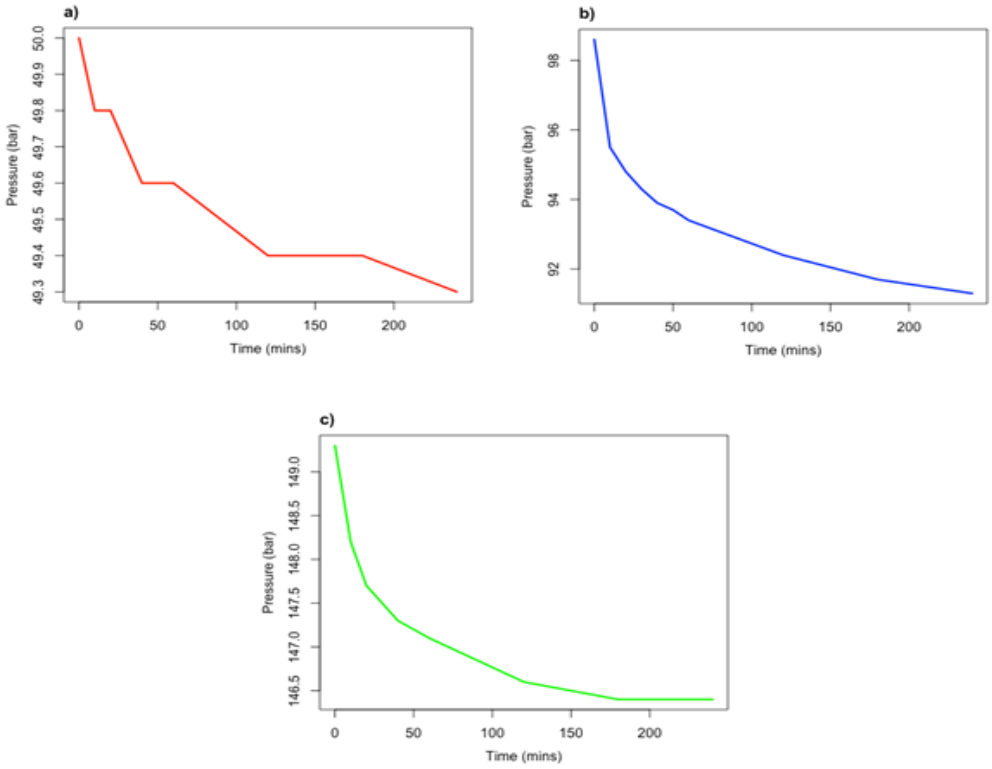

Figure (6.3) *a) Pressure maintained within the vessel at a starting pressure of 50 bar for a period of 6 hours. b) Pressure maintained within the vessel at a starting pressure of 100 bar for a period of 6 hours. c) Pressure maintained within the vessel at a starting pressure of 150 bar for a period of 6 hours. NOTE: y-axis for a, b and c do not begin at 0*

6.4 Results

6.4.1 Effect of salinity and pH on biomass accumulation under 50-bar hydrostatic pressure

To measure the relationship between concentrations of NaCl [wt/vol], variations in pH and simulated hydrostatic pressure on microbial propagation, *H. hydrothermalis* was cultured under a range of salinities and pH at 50-bar and atmospheric pressure. When cultured under the hydrostatic pressure of 50-bar, *H. hydrothermalis* displayed growth up to and including the highest salinity used in this study (9.14% NaCl [wt/vol]). Biomass accumulation was additionally measured at all salinities tested under atmospheric conditions, however mean OD_{600} values were consistently higher under 50-bar simulated hydrostatic pressure than atmospheric pressure (excluding pH 8 salinities 8.34 and 8.80% NaCl [wt/vol], with neither shown to display statistically significantly differences between growth conditions) (Table. 6.1).

Mean OD_{600} values obtained at pH 8 under atmospheric pressure and 50-bar pressure displayed highly significant strong negative linear correlation with salinity (r = -0.565, $p < 0.001$ and r = -0.79, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient) (Figure 6.4). Two-sample independent Students t-tests of pH 8 data revealed differences in OD_{600} values between pressure conditions were shown to be statistically significant at 4.18 and 4.64 % NaCl [wt/vol] (Table. 6.1). Pearson's product-moment correlation was performed to provide a measure of the direction of linear correlation between NaCl concentration [wt/vol] and mean OD_{600}. At pH 7, correlation between mean OD_{600} values and salinity was shown to be significantly moderate negative (r = -0.305, $p < 0.05$; Pearsons product-moment correlation coefficient) and significantly weak negative linear correlation at atmospheric pressure (r = -0.274, $p < 0.05$; Pearsons product-moment correlation coefficient) (Figure 6.5). Two-sample t-test of pH 7 data reveals significant differences between 1.87, 2.33, 3.25, 3.72, 4.64, 6.95, 7.41, 7.87% NaCl [wt/vol] (Table. 6.2). Under pH 6 conditions, correlation between mean OD_{600} and salinity was shown to display a highly significant moderate negative linear correlation for both atmospheric and 50-bar pressure culture conditions (r = -0.493, $p < 0.001$ and r = -0.434, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient) (Figure 6.6). Two-sample t-tests of pH 6 data show significant differences between 1.41,

1.87, 2.33, 3.25, 3.72, 4.18, 6.03, 6.49, 6.95, 7.41, 7.87, 8.34, 9.14% NaCl [wt/vol] (Table. 6.3).

Table (6.1) *Mean and Standard Deviation ± (n = 4) of the mean OD_{600} values of* Halomonas hydrothermalis *cultured at pH 8 under simulated hydrostatic pressure at 50-bar and at atmospheric pressure under a range of salinities (% NaCl [wt/vol] (n = 19), and t-test results comparing the mean values. Mean values shown to be higher are bold for the pressure respective condition.*

pH 8 NaCl (%)	50-bar	control	*P*
0.95	**0.226 ± 0.08**	0.206 ± 0.1	$t(6) = 0.3, p = 0.847$
1.41	**0.303 ±0.11**	0.139 ± 0.1	$t(5) = 2.1, p = 0.179$
1.87	**0.289 ± 0.11**	0.095 ± 0.04	$t(6) = 3.4, p = 0.065$
2.33	**0.195 ± 0.05**	0.138 ± 0.09	$t(6) = 1.1, p = 0.410$
2.79	**0.191 ± 0.05**	0.153 ± 0.12	$t(6) = 0.6, p = 0.672$
3.25	**0.195 ± 0.04**	0.144 ± 0.07	$t(5) = 1.1, p = 0.410$
3.72	**0.246 ± 0.08**	0.104 ± 0.06	$t(5) = 2.8$, p = 0.127
4.18	**0.207 ± 0.02**	0.093 ± 0.03	$t(6) = 6.5$, **$p < 0.01$**
4.64	**0.224 ± 0.03**	0.058 ± 0.01	$t(6) = 11.8$, **$p < 0.001$**
5.1	**0.150 ± 0.01**	0.081 ± 0.04	$t(6) = 3.3, p = 0.064$
5.56	**0.157 ± 0.02**	0.126 ± 0.04	$t(6) = 1.5, p = 0.301$
6.03	**0.144 ± 0.02**	0.105 ± 0.02	$t(5) = 2.5, p = 0.152$
6.49	**0.131 ± 0.04**	0.083 ± 0.02	$t(6) = 2.2, p = 0.176$
6.95	**0.115 ± 0.01**	0.077 ± 0.04	$t(6) = 1.9, p = 0.203$
7.41	**0.105 ± 0.01**	0.062 ± 0.04	$t(5) = 1.7$, p = 0.261
7.87	0.033 ± 0.02	**0.036 ± 0.03**	$t(6) = -0.2, p = 0.902$
8.34	**0.055 ± 0.06**	0.051 ± 0.03	$t(6) = 0.1, p = 0.902$
8.8	0.027 ± 0.03	**0.049 ± 0.03**	$t(5) = -1.1, p = 0.410$
9.14	**0.078 ± 0.01**	0.032 ± 0.02	$t(6) = 3.5, p = 0.065$

Table (6.2) *Mean and Standard Deviation ± (n = 4) of the mean OD_{600} values of* Halomonas hydrothermalis *cultured at pH 7 under simulated hydrostatic pressure at 50-bar and at atmospheric pressure under a range of salinities (% NaCl [wt/vol] (n = 19), and t-test results comparing the mean values. Mean values shown to be higher are bold for the pressure respective condition.*
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

pH 7 **NaCl (%)**	**50-bar**	**control**	***P***
0.95	**0.170 ± 0.05**	0.131 ± 0.05	$t(6) = 1.1, p = 0.315$
1.41	**0.189 ± 0.03**	0.116 ± 0.05	$t(5) = 2.3, p = 0.087$
1.87	**0.245 ± 0.06**	0.078 ± 0.04	$t(5) = 4.2,$ **$p < 0.05$**
2.33	**0.288 ± 0.09**	0.086 ± 0.04	$t(6) = 4.1,$ **$p < 0.05$**
2.79	**0.254 ± 0.1**	0.096 ± 0.06	$t(6) = 2.8, p = 0.057$
3.25	**0.225 ± 0.03**	0.116 ± 0.04	$t(6) = 4.2,$ **$p < 0.05$**
3.72	**0.255 ± 0.02**	0.090 ± 0.03	$t(6) = 10.9,$ **$p < 0.001$**
4.18	**0.225 ± 0.02**	0.162 ± 0.09	$t(5) = 1.2, p = 0.295$
4.64	**0.225 ± 0.06**	0.091 ± 0.04	$t(6) = 3.7,$ **$p < 0.05$**
5.1	**0.184 ± 0.05**	0.100 ± 0.06	$t(5) = 2.1, p = 0.106$
5.56	**0.188 ± 0.05**	0.097 ± 0.05	$t(6) = 2.6, p = 0.058$
6.03	**0.221 ± 0.08**	0.103 ± 0.03	$t(6) = 2.7,$ p = 0.058
6.49	**0.310 ± 0.3**	0.093 ± 0.04	$t(3) = 1.4, p = 0.274$*
6.95	**0.226 ± 0.06**	0.088 ± 0.04	$t(5) = 3.6,$ **$p < 0.05$**
7.41	**0.175 ± 0.06**	0.071 ± 0.02	$t(5) = 3.4,$ **$p < 0.05$**
7.87	**0.148 ± 0.03**	0.050 ± 0.01	$t(6) = 7,$ **$p < 0.001$**
8.34	**0.150 ± 0.02**	0.097 ± 0.04	$t(6) = 2.6, p = 0.058$
8.8	**0.121 ± 0.01**	0.076 ± 0.04	$t(6) = 2.4, p = 0.073$
9.14	**0.116 ± 0.03**	0.073 ± 0.02	$t(6) = 2.9, p = 0.057$

Table (6.3) *Mean and Standard Deviation ± (n = 4) of the mean OD_{600} values of* Halomonas hydrothermalis *cultured at pH 6 under simulated hydrostatic pressure at 50-bar and at atmospheric pressure under a range of salinities (% NaCl [wt/vol] (n = 19), and t-test results comparing the mean values. Mean values shown to be higher are bold for the pressure respective condition.*
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

pH 6 NaCl (%)	50-bar	control	*P*
0.95	**0.121 ± 0.06**	0.115 ± 0.05	$t(5) = 0.1, p = 0.889$
1.41	**0.219 ± 0.05**	0.098 ± 0.04	$t(6) = 3.9,$ **$p < 0.05$**
1.87	**0.193 ± 0.02**	0.086 ± 0.05	$t(6) = 4,$ **$p < 0.05$**
2.33	**0.219 ± 0.04**	0.090 ± 0.04	$t(5) = 4.5,$ **$p < 0.05$**
2.79	**0.172 ± 0.03**	0.106 ± 0.05	$t(5) = 2.1, p = 0.107$
3.25	**0.227 ± 0.05**	0.117 ± 0.05	$t(6) = 3.2,$ **$p < 0.05$**
3.72	**0.216 ± 0.08**	0.069 ± 0.01	$t(3) = 3.7,$ **$p < 0.05$***
4.18	**0.236 ± 0.03**	0.092 ± 0.05	$t(6) = 5.2,$ **$p < 0.05$**
4.64	**0.190 ± 0.05**	0.101 ± 0.05	$t(5) = 2.4, p = 0.076$
5.1	**0.162 ± 0.03**	0.107 ± 0.05	$t(6) = 1.7, p = 0.139$
5.56	**0.126 ± 0.02**	0.074 ± 0.05	$t(6) = 1.8, p = 0.139$
6.03	**0.174 ± 0.01**	0.078 ± 0.04	$t(5) = 4.4,$ **$p < 0.05$**
6.49	**0.159 ± 0.03**	0.083 ± 0.03	$t(6) = 3.7,$ **$p < 0.05$**
6.95	**0.123 ± 0.01**	0.055 ± 0.03	$t(5) = 3.8,$ **$p < 0.05$**
7.41	**0.124 ± 0.01**	0.050 ± 0.03	$t(6) = 5.3,$ **$p < 0.05$**
7.87	**0.179 ± 0.06**	0.048 ± 0.01	$t(3) = 4.1,$ **$p < 0.05$**
8.34	**0.160 ± 0.05**	0.061 ± 0.03	$t(6) = 3,$ **$p < 0.05$**
8.8	**0.110 ± 0.03**	0.04 ± 0.005	$t(2) = 4, p = 0.076$*
9.14	**0.128 ± 0.03**	0.036 ± 0.01	$t(3) = 0.04,$ **$p < 0.05$***

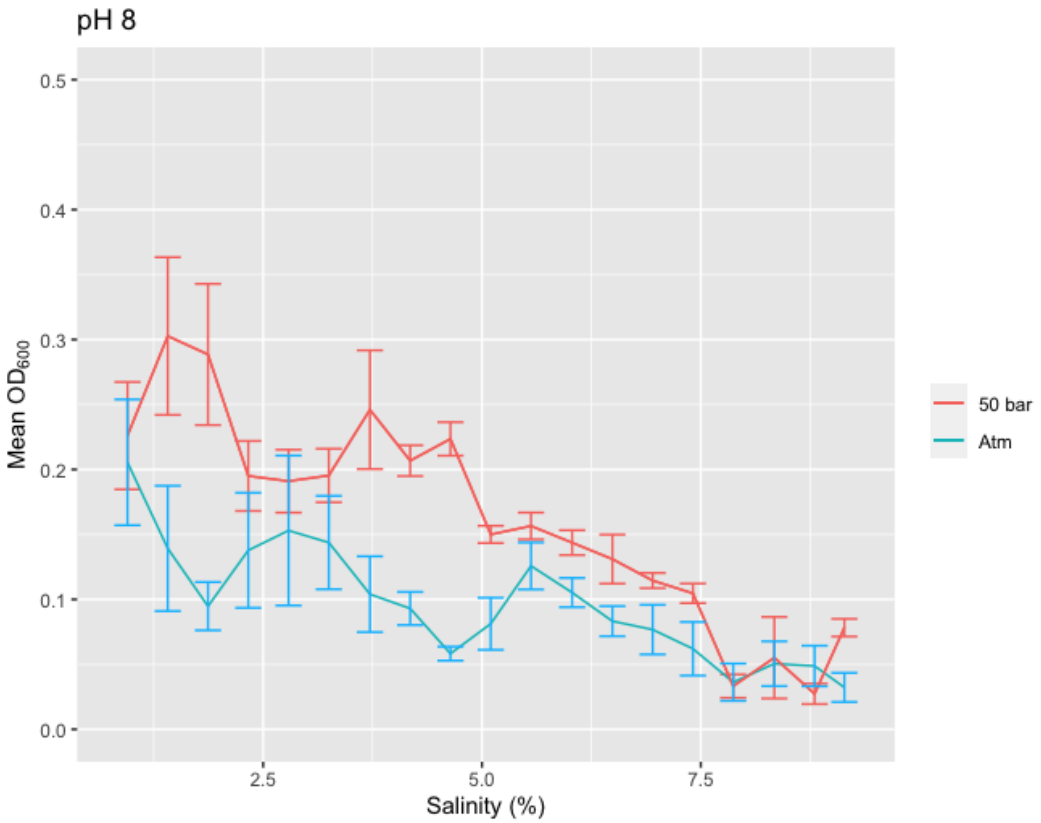

Figure (6.4) ***Graph displaying mean OD_{600} values obtained under pH 8 and a range of salinities (% NaCl [wt/vol] (n = 19) for* H. hydrothermalis *at 50-bar pressure and atmospheric pressure.***

Data presented as mean OD_{600} values ± standard error of the means (SE) (n = 4). H. hydrothermalis *displayed significantly strong negative linear correlation with salinity (r = -0.565, $p < 0.001$ and r = -0.79, $p < 0.001$ respectively; Pearsons product-moment correlation coefficient).*

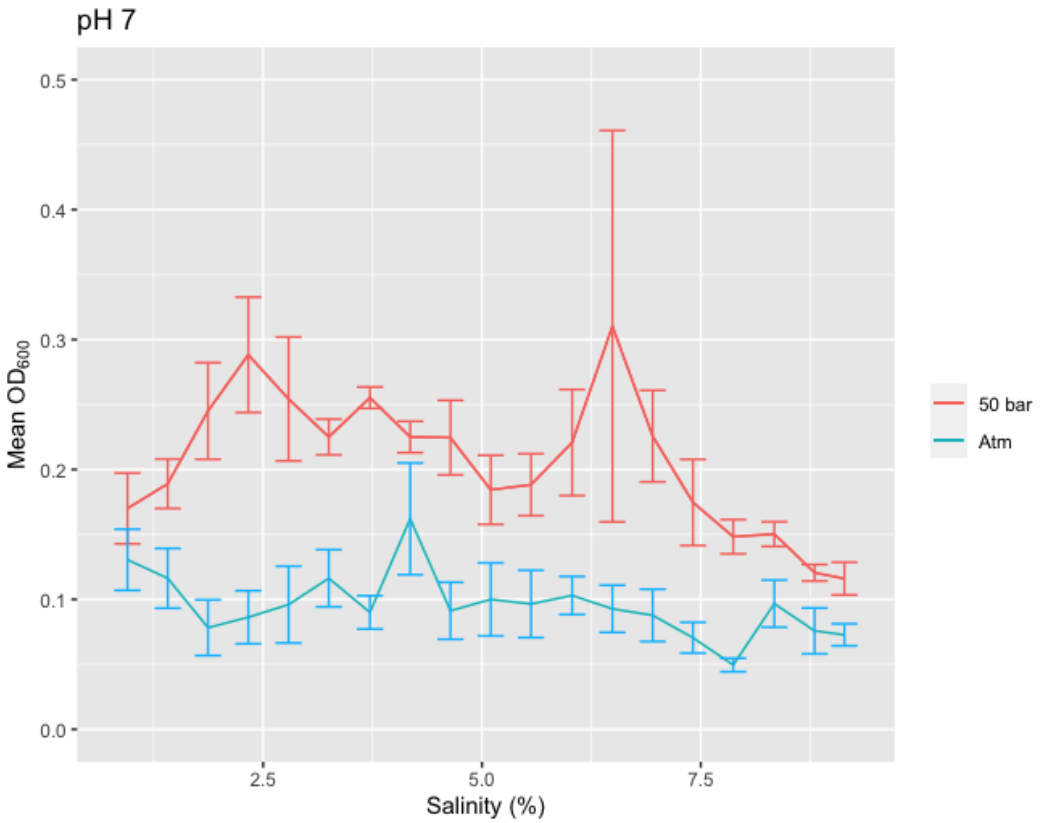

Figure (6.5) ***Graph displaying mean OD_{600} values obtained under pH 7 and a range of salinities (% NaCl [wt/vol] (n = 19) for* H. hydrothermalis *at 50-bar pressure and atmospheric pressure.***

Data presented as mean OD_{600} values ± standard error of the means (SE) (n = 4). H. hydrothermalis *displayed significantly moderate negative correlation between mean OD_{600} values at 50-bar pressure and salinity ($r = -0.305$, $p < 0.05$; Pearsons product-moment correlation coefficient), and significantly weak negative correlation at atmospheric pressure ($r = -0.274$, $p < 0.05$; Pearsons product-moment correlation coefficient).*

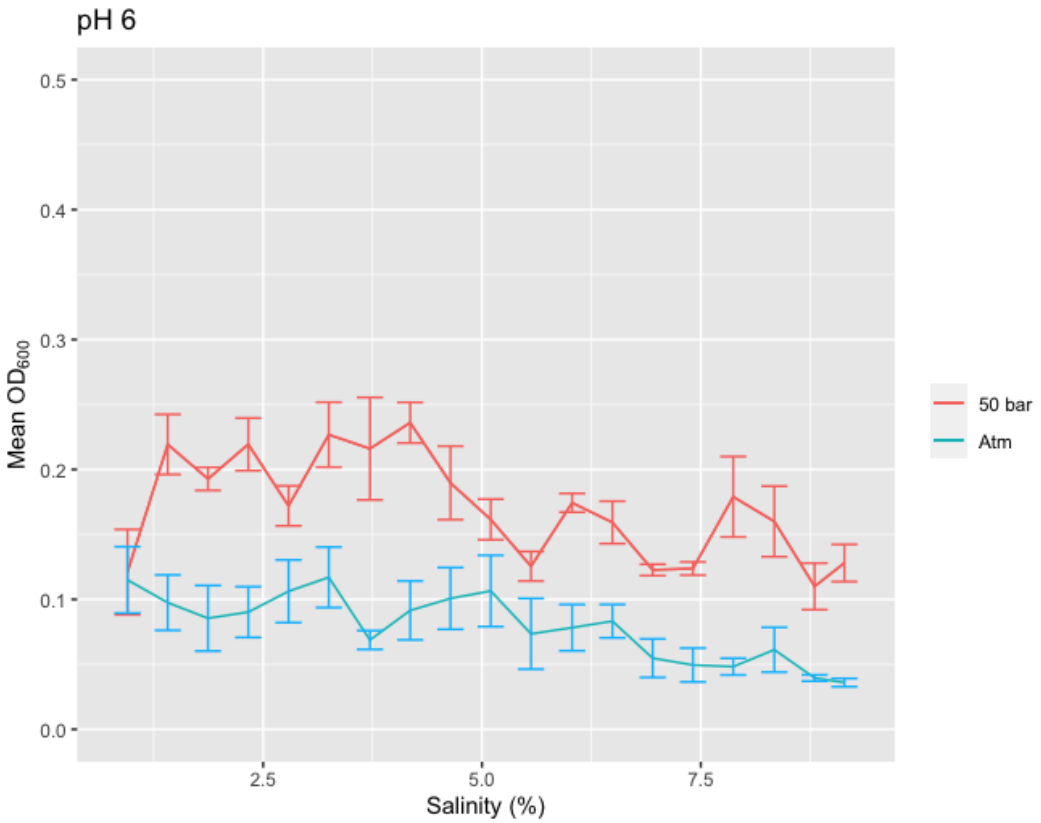

Figure (6.6) ***Graph displaying mean OD_{600} values obtained under pH 6 and a range of salinities (% NaCl [wt/vol] (n = 19) for* H. hydrothermalis *at 50-bar pressure and atmospheric pressure.*** *//* Data presented as mean OD_{600} values ± standard error of the means (SE) (n = 4).* H. hydrothermalis *displayed highly significantly moderate negative correlation for both 50-bar and atmospheric pressure culture conditions (r = -0.434, p < 0.001 and r = -0.493, p < 0.001 respectively; Pearsons product-moment correlation coefficient).*

6.4.2 Effect of salinity and pH on biomass accumulation under 150-bar hydrostatic pressure

To further measure the effect of simulated hydrostatic pressure on microbial propagation under combined stresses, OD_{600} values were obtained under pressure conditions of 150-bar and atmospheric pressures. Under culture conditions of 150-bar, *H. hydrothermalis* displayed cell division consistently higher than those measured under atmospheric conditions at pH 6 and 7. When cultured at pH 8, OD_{600} values at salinities 0.95, 1.41, 2.33, 2.79, and 3.25% NaCl [wt/vol] were higher under atmospheric pressure conditions, however the differences between mean OD_{600} values at these salinities were not shown to be statistically significant (Table. 6.4).

Mean OD_{600} values obtained under pH 8 culture conditions at atmospheric pressure displayed a highly significant negative linear correlation with salinity (r = -0.457, $p < 0.001$; Pearsons product-moment correlation coefficient), however, values obtained at 150-bar pressure and pH 8 conditions were not shown to be significantly correlated with salinity (r = -0.052, p = 0.684; Pearsons product-moment correlation coefficient) (Figure 6.7). Significant differences as seen by a two-sample independent Students t-test between OD_{600} values at pH 8 were seen only at 4.18% [wt/vol] (Table. 6.4). Pearson's product-moment correlation was performed to provide a measure of the direction of linear correlation between NaCl concentration [wt/vol] and mean OD_{600}. At pH 7, mean OD_{600} values obtained under atmospheric pressure displayed highly significant moderate negative linear correlation with salinity (r = -0.457 $p <$ 0.001; Pearsons product-moment correlation coefficient), and 150-bar hydrostatic pressure displayed highly significant negative linear correlation with salinity (r = -0.599, $p < 0.001$; Pearsons product-moment correlation coefficient) (Figure 6.8). Two-sample independent Students t-test of pH 7 data revealed significant differences between OD_{600} values at 1.87, 2.33, 2.79, 3.25, 3.72, 5.10, 5.56, 6.49, 7.41, 8.80 and 9.14% [wt/vol] (Table. 6.5). Under pH 6 culture conditions, mean OD_{600} values obtained under atmospheric pressure displayed highly significant negative linear correlation with salinity (r = -0.757, $p < 0.001$; Pearsons product-moment correlation coefficient), and under 150-bar simulated hydrostatic pressure there was significantly weak negative linear correlation between mean OD_{600} values and salinity (r = -0.247, $p < 0.05$; Pearsons product-moment correlation coefficient) (Figure 6.8). Two-sample t-tests of pH 6 data show significant

differences between 3.72, 5.56, 6.03, 7.41, 8.34, 8.80 and 9.14% [wt/vol] for cultures at pH 6 (Table 6.6).

Table (6.4) *Mean and Standard Deviation ± (n = 4) of the mean OD_{600} values of* Halomonas hydrothermalis *cultured at pH 8 under simulated hydrostatic pressure at 150-bar and at atmospheric pressure under a range of salinities (% NaCl [wt/vol] (n = 19), and t-test results comparing the mean values. Mean values shown to be higher are bold for the pressure respective condition.*
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

pH 8 NaCl (%)	150-bar	control	*P-value*
0.95	0.092 ± 0.02	**0.266 ± 0.17**	$t(3) = -2, p = 0.245$*
1.41	0.092 ± 0.03	**0.321 ± 0.22**	$t(2) = -1.8, p = 0.366$*
1.87	**0.152 ± 0.03**	0.148 ± 0.14	$t(5) = 0.05, p = 0.957$
2.33	0.189 ± 0.01	**0.254 ± 0.22**	$t(3) = -0.6, p = 0.808$*
2.79	0.198 ± 0.02	**0.220 ± 0.2**	$t(3) = -0.2, p = 0.881$*
3.25	0.188 ± 0.05	**0.237 ± 0.12**	$t(6) = -0.7, p = 0.722$
3.72	**0.190 ± 0.01**	0.083 ± 0.05	$t(5) = 3.3, p = 0.100$
4.18	**0.221 ± 0.04**	0.069 ± 0.03	$t(6) = 6.5, \mathbf{p < 0.05}$
4.64	**0.187 ± 0.01**	0.086 ± 0.05	$t(6) = 4.2, p = 0.057$
5.1	**0.236 ± 0.04**	0.153 ± 0.15	$t(5) = 1, p = 0.613$
5.56	**0.199 ± 0.01**	0.182 ± 0.08	$t(5) = 0.4, p = 0.879$
6.03	**0.182 ± 0.01**	0.168 ± 0.08	$t(4) = 0.2, p = 0.881$
6.49	**0.258 ± 0.08**	0.137 ± 0.04	$t(6) = 2.7, p = 0.108$
6.95	**0.194 ± 0.06**	0.094 ± 0.02	$t(5) = 3.3, p = 0.100$
7.41	**0.138 ± 0.01**	0.130 ± 0.04	$t(5) = 0.4, p = 0.879$
7.87	**0.171 ± 0.04**	0.074 ± 0.01	$t(2) = 4, p = 0.118$*
8.34	**0.137 ± 0.02**	0.100 ± 0.02	$t(5) = 2.5, p = 0.119$
8.8	**0.106 ± 0.03**	0.064 ± 0.02	$t(5) = 2.5, p = 0.118$
9.14	**0.104 ± 0.01**	0.066 ± 0.02	$t(5) = 2.9, p = 0.108$

Table (6.5) *Mean and Standard Deviation ± (n = 4) of the mean OD_{600} values of* Halomonas hydrothermalis *cultured at pH 7 under simulated hydrostatic pressure at 150-bar and at atmospheric pressure under a range of salinities (% NaCl [wt/vol] (n = 19), and t-test results comparing the mean values. Mean values shown to be higher are bold for the pressure respective condition.*
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

pH 7 **NaCl (%)**	**150-bar**	**control**	***P-value***
0.95	**0.257 ± 0.01**	0.159 ± 0.08	$t(3) = 2.3, p = 0.137$*
1.41	**0.249 ± 0.07**	0.170 ± 0.05	$t(5) = 1.9, p = 0.137$
1.87	**0.394 ± 0.07**	0.092 ± 0.01	$t(2) = 8,$ **$p < 0.05$***
2.33	**0.376 ± 0.07**	0.125 ± 0.07	$t(6) = 5.2,$ **$p < 0.01$**
2.79	**0.448 ± 0.10**	0.081 ± 0.03	$t(5) = 7.6,$ **$p < 0.01$**
3.25	**0.355 ± 0.05**	0.175 ± 0.02	$t(6) = 6.2,$ **$p < 0.01$**
3.72	**0.188 ± 0.01**	0.115 ± 0.04	$t(6) = 3.3,$ **$p < 0.05$**
4.18	**0.337 ± 0.13**	0.153 ± 0.01	$t(2) = 2.5, p = 0.137$*
4.64	**0.190 ± 0.04**	0.130 ± 0.06	$t(5) = 1.5, p = 0.203$
5.1	**0.293 ± 0.03**	0.096 ± 0.04	$t(5) = 8.1,$ **$p < 0.001$**
5.56	**0.232 ± 0.04**	0.111 ± 0.03	$t(6) = 5.3,$ **$p < 0.01$**
6.03	**0.242 ± 0.05**	0.115 ± 0.11	$t(5) = 1.9, p = 0.137$
6.49	**0.236 ± 0.07**	0.090 ± 0.02	$t(6) = 4.1,$ **$p < 0.05$**
6.95	**0.238 ± 0.14**	0.072 ± 0.02	$t(3) = 2.3, p = 0.103$*
7.41	**0.145 ± 0.02**	0.079 ± 0.02	$t(6) = 5.3,$ **$p < 0.01$**
7.87	**0.151 ± 0.03**	0.081 ± 0.06	$t(6) = 2.2, p = 0.117$
8.34	**0.166 ± 0.01**	0.110 ± 0.05	$t(5) = 2, p = 0.137$
8.8	**0.143 ± 0.02**	0.055 ± 0.03	$t(5) = 4.3,$ **$p < 0.05$**
9.14	**0.191 ± 0.05**	0.052 ± 0.02	$t(6) = 4.8,$ **$p < 0.01$**

Table (6.6) *Mean and Standard Deviation ± (n = 4) of the mean OD_{600} values of* Halomonas hydrothermalis *cultured at pH 6 under simulated hydrostatic pressure at 150-bar and at atmospheric pressure under a range of salinities (% NaCl [wt/vol] (n = 19), and t-test results comparing the mean values. Mean values shown to be higher are bold for the pressure respective condition.*
** Denotes instances the variance was shown to unequal (Bartletts test) in which case a Welchs t-test was performed.*

pH 6 **NaCl (%)**	**150-bar**	**control**	***P-value***
0.95	0.119 ± 0.03	0.119 ± 0.01	$t(6) = 0, p = 1.00$
1.41	**0.183 ± 0.06**	0.156 ± 0.04	$t(6) = 0.8, p = 0.527$
1.87	**0.211 ± 0.12**	0.120 ± 0.01	$t(2) = 1.3, p = 0.415$*
2.33	**0.252 ± 0.04**	0.134 ± 0.04	$t(6) = 2.7, p = 0.067$
2.79	**0.201 ± 0.08**	0.172 ± 0.03	$t(6) = 0.7, p = 0.572$
3.25	**0.238 ± 0.08**	0.156 ± 0.04	$t(5) = 1.5, p = 0.270$
3.72	**0.182 ± 0.10**	0.080 ± 0.02	$t(5) = 6.2$, **$p < 0.05$**
4.18	**0.212 ± 0.07**	0.111 ± 0.01	$t(3) = 2.8, p = 0.096$*
4.64	**0.165 ± 0.04**	0.150 ± 0.01	$t(6) = 0.8, p = 0.474$
5.1	**0.277 ± 0.11**	0.116 ± 0.05	$t(6) = 2.6, p = 0.058$
5.56	**0.231 ± 0.04**	0.120 ± 0.03	$t(5) = 4.3$, **$p < 0.05$**
6.03	**0.221 ± 0.06**	0.084 ± 0.04	$t(6) = 3.8$, **$p < 0.05$**
6.49	**0.254 ± 0.07**	0.061 ± 0.004	$t(2) = 5.1$, p = 0.067
6.95	**0.250 ± 0.08**	0.038 ± 0.01	$t(3) = 5, p = 0.053$
7.41	**0.178 ± 0.04**	0.038 ± 0.03	$t(6) = 5.6$, **$p < 0.05$**
7.87	**0.164 ± 0.06**	0.041 ± 0.01	$t(2) = 3.8, p = 0.093$*
8.34	**0.117 ± 0.04**	0.045 ± 0.01	$t(3) = 3.5$, **$p < 0.05$***
8.8	**0.082 ± 0.02**	0.039 ± 0.02	$t(6) = 2.8$, **$p < 0.05$**
9.14	**0.094 ± 0.03**	0.022 ± 0.01	$t(3) = 4.2$, **$p < 0.05$***

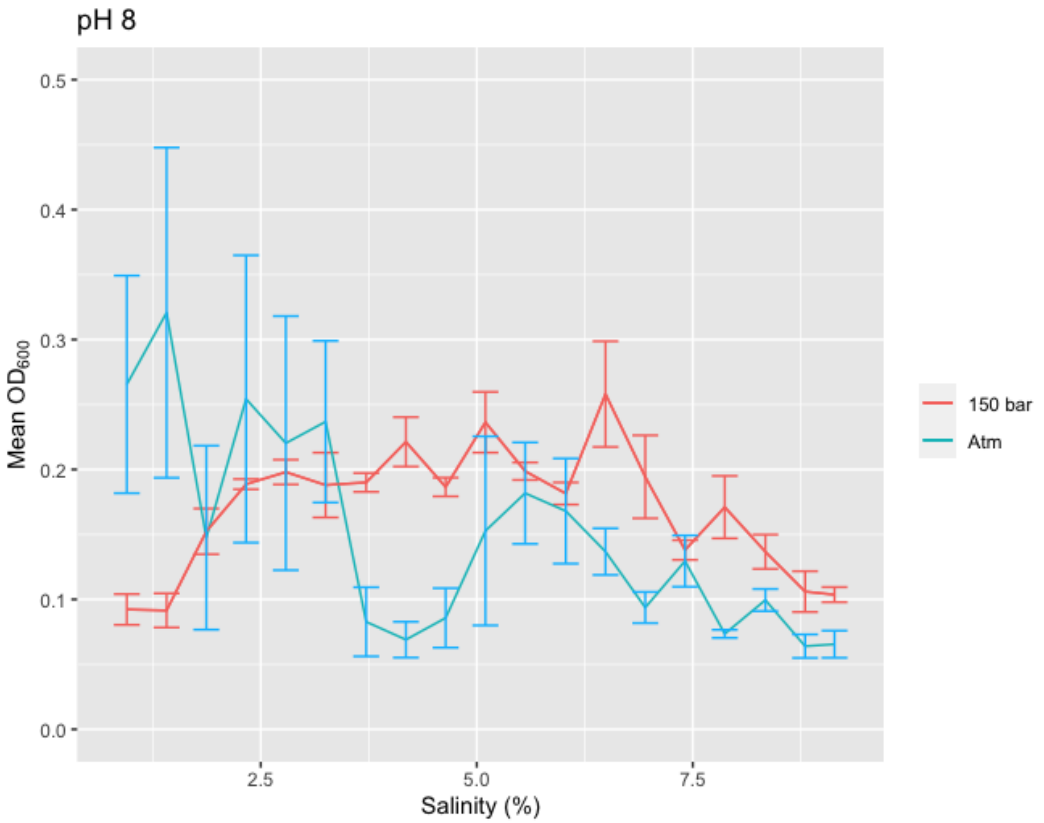

Figure (6.7) ***Graph displaying mean OD_{600} values obtained under pH 8 and a range of salinities (% NaCl [wt/vol] (n = 19) for* H. hydrothermalis *at 150-bar pressure and atmospheric pressure.***

Data presented as mean OD_{600} values ± standard error of the means (SE) (n = 4). H. hydrothermalis *displayed highly significant negative correlation with salinity (r = -0.457, p < 0.001; Pearsons product-moment correlation coefficient). 150-bar pressure conditions were not shown to be correlated with salinity (r = -0.0.52, p = 0.684; Pearsons product-moment correlation coefficient).*

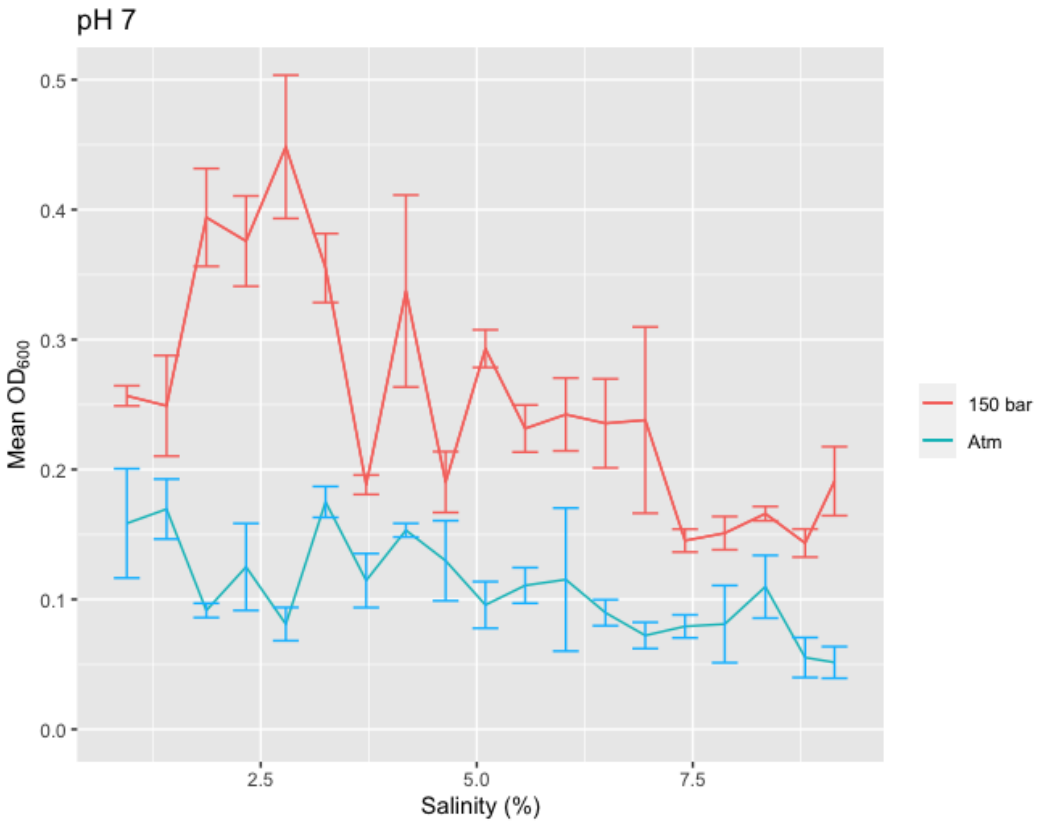

Figure (6.8) ***Graph displaying mean OD_{600} values obtained under pH 7 and a range of salinities (% NaCl [wt/vol] (n = 19) for* H. hydrothermalis *at 150-bar pressure and atmospheric pressure.***

Data presented as mean OD_{600} values ± standard error of the means (SE) (n = 4). H. hydrothermalis *displayed highly significant negative linear correlation with salinity under atmospheric pressure and 150-bar pressure (r = -0.457 p ¡ 0.001 and r = -0.599, p ¡ 0.001 respectively; Pearsons product-moment correlation coefficient).*

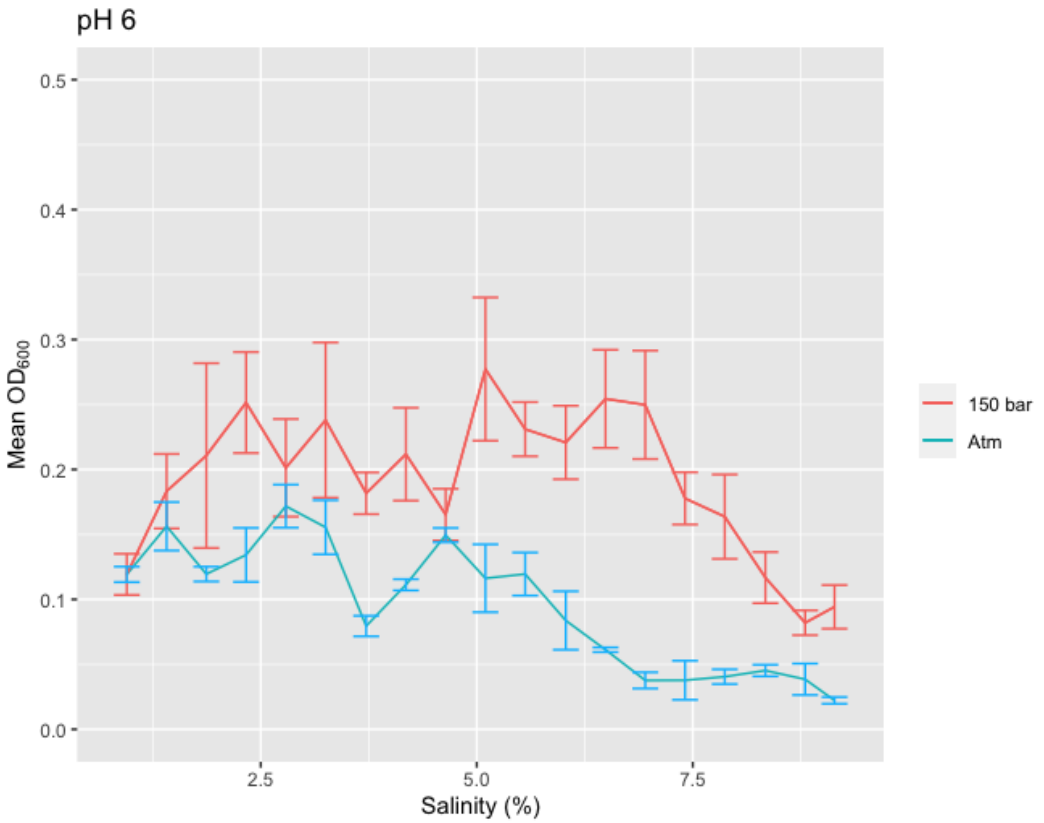

Figure (6.9) ***Graph displaying mean OD_{600} values obtained under pH 6 and a range of salinities (% NaCl [wt/vol] (n = 19) for* H. hydrothermalis *at 150-bar pressure and atmospheric pressure.***

Data presented as mean OD_{600} values ± standard error of the means (SE) (n = 4). H. hydrothermalis *displayed highly significant negative correlation with salinity and atmospheric pressure (r = -0.757, p < 0.001; Pearsons product-moment correlation coefficient). 150-bar pressure conditions and salinity displayed significantly weak negative correlation (r = -0.247, p < 0.05; Pearsons product-moment correlation coefficient).*

6.5 Discussion

Pressure as a parameter for life ranges from the atmospheric pressure of ∼ 1 bar at sea level to the deep ocean pressures exceeding 1000 bar (Mota et al., 2013; Picard and Daniel, 2013). Some environments exhibit pressures lower than atmospheric levels, such as high-altitude mountains and low Earth orbit, though these are not thought to affect microbial viability (Merino et al., 2019), however data is scarce and the affect of low pressure environments on microbial survivability is not understood. High-pressure environments are ubiquitous on Earth, with possibly the largest ecosystem represented by the deep-sea piezosphere (Abe, 2007; Bartlett, 1999; Jannasch and Taylor., 1984), and communities of microorganisms have been detected as far as 3500 m below the surface of the Earth in a bore hole drilled in granite (Szewzyk et al., 1994). It is clear that the majority of microbial life on Earth occupies habitats that experience higher than atmospheric pressures and thereby require the necessary adaptations to deal with such environmental constraints. Many high-pressure environments are subject to additional environmental stresses, such as deep-sea hydrothermal vents systems which are known to display variations of additional environmental factors such as pH (Holden and Baross, 1995; Jebbar et al., 2015). This study aimed to quantify the combined effect of stresses of salinity and pH under simulated hydrostatic pressures on the model organism *H. hydrothermalis*, isolated from deep-sea hydrothermal fluid at a depth of 2580 m (Kaye et al., 2004). Experimental parameters of pH and salinity were chosen due to their established limits to life individually and prevalence in natural habitats (Blum et al., 2009; Schleper et al., 1996; Suzuki et al., 2014). Hydrostatic pressure was selected for this study to provide a more robust understanding of the effect of physical constraints on microbial growth under a combination of other environmental stresses.

The work presented in this chapter demonstrates that when cultivated over a range of salinities and pH values, *H. hydrothermalis* displays greater biomass accumulation by OD_{600} measurement under simulated hydrostatic pressures than when cultured under atmospheric pressure conditions. This effect is observed most prominently in culture conditions of pH 7 and pH 6, where the majority of growth yields under hydrostatic pressures of 50-bar and 150-bar were greater than growth yields under atmospheric pressure. The effect is less pronounced under pH 8 culture conditions; however, a higher

proportion of greater mean OD_{600} values are still oberved under simulated hydrostatic pressure conditions than under atmospheric pressure. This is particularly true for mean OD_{600} values obtained under higher salinities at 150-bar simulated hydrostatic pressure. These data demonstrate an increase of 100-bar hydrostatic pressure reduced the detrimental effect of salinity on growth of *H. hydrothermalis*. Additionally, growth was higher under 150-bar pressure when compared with atmospheric conditions upon reaching the optimal salinity range for *H. hydrothermalis*, but predominantly greater under atmospheric conditions before reaching this point.

These pressure effects were further demonstrated when cultivated at neutral pH 7, where significant differences in mean OD_{600} values are seen throughout the salinity range tested in this study under both 50-bar and 150-bar simulated hydrostatic pressures. This may be due to increased membrane rigidity under higher pressures. Contrary to the concept that organisms experience a loss of membrane fluidity under higher pressures, and therefore have reduced capacity to cope with an increase in osmotic stress (Huang et al., 2016), the naturally high-pressure environment from which *H. hydrothermalis* was isolated (see Chapter 3), and the increased growth under saline conditions suggests that for this particular microbe it is atmospheric pressure that can be considered the extreme parameter.

Under pH 6 culture conditions, the results again show that growth values under high pressure conditions are significantly higher than those under atmospheric conditions for the majority of salinities tested, with a notable trend of increased growth under saline conditions at increased pressures. Increased growth under mildly acid conditions may be a result of *H. hydrothermalis* being better able to maintain a near neutral intracellular pH due to increased membrane rigidity. High pressure is known to share common adaptations to low temperature extremes through changes in membrane composition (Casadei et al., 2002; Siliakus et al., 2017). Indeed, pH, low temperature and pressure are known to be common physicochemical parameters that affect membrane integrity, causing increased lipid packing and a loss of permeability (Oger and Jebbar, 2010; Siliakus et al., 2017). The loss of membrane permeability and resulting decrease in ion-permeability may be directly affecting the mechanisms responsible for dealing with extremes in pH, resulting in an expansion of pH growth limits at mildly acid conditions that under atmospheric pressures prove detrimental. As pH is known to be an important factor in shaping microbial community composition (Merino et al., 2019), it is clear from these data that the additional physical constraint

of high pressure also plays a role in influencing microbial growth in these other environmental extremes.

The cultures employed in this chapter were able to better propagate over a wider range of salinities under simulated hydrostatic pressures than under atmospheric pressure conditions. Additionally, this study demonstrates an increased capacity to deal with previously established limits in pH for the model organism (see Chapter 4) under high-pressure conditions than under atmospheric pressure. These findings show that, under a combination of stress conditions, both optimal, sub- and supra-optimal, the physical environmental constraint of hydrostatic pressure significantly expands the limits of life under additional stress parameters. These data further demonstrate the necessity to assess the habitability of natural environments with a deeper understanding of the interplay between concomitant physico-chemical parameters. Even with our current knowledge of microbial adaptation to extremes imposed in isolation, a more robust understanding of the combined effect of simultaneously occurring stresses is crucial in determining the habitability of a given environment. The recognition that there are more instances of life occupying habitats that are subject to greater than atmospheric pressures is significant in determining what constitutes an extreme environmental parameter. It would be appropriate to consider higher than atmospheric pressures not as an extreme parameter itself but rather a boundary space occupied by a higher proportion of life than those that operate outside that space, and consequently not an extreme condition for the majority of life on Earth. Additional to this, an organisms ability to propagate within this boundary space, or indeed specifically only within this space, further defines the extremity of such an environment in light of microbial growth preferences.

To determine what constitutes an extreme condition one must consider the environmental stresses imposed on organisms in a given habitat. In the case of *H. hydrothermalis*, the physicochemical parameters that characterise hydrothermal vent systems should be considered when defining extreme environmental variables. Indeed, deep-sea hydrothermal vents are ideal ecosystems in the pursuit of this investigation as they are subject to extreme variations in temperatures (from 2 to4°C up to 450°C), acidic pH (Ding et al., 2005; Tan et al., 2017) and isolated are known to display broad salinity ranges [see organisms isolated in Kaye et al. (2004) and Dalmasso et al. (2016). Further to the data reported in Chapters 4 and 5, these results provide additional applications to astrobiology.

For example, Jupiters moon Europa and Saturns moon Enceladus are suggested to experience Earth-like deep-sea pressures and moderate temperatures, which make them prime candidates in the search for life on extra planetary bodies (Greenberg et al., 1998; Hsu et al., 2015; Sotin and Tobie, 2004; Tyler, 2008). The data presented here can be used in assessing the theoretical habitability of these bodies.

6.5.1 Limitations

Though the experimental work performed in this chapter allowed for the direct comparison of growth yields of *H. hydrothermalis* cultivated at atmospheric pressure and simulated hydrostatic pressure, use of high-pressure equipment is not without its limitations. The use of high-pressure equipment involves technical restrictions that limit the ability to collect microbial growth measurements during periods of pressurisation. This limited the duration of high-pressure experiments to the established timeline of microbial growth for the model organism and did not allow for experimental protocol to cover the low temperatures experienced in the natural habitat that would result in significantly slower growth.

6.5.2 Future Work

The collaborative work for the chapter has set in to place the necessary equipment and setup to allow for further work to be carried out between The UK Centre for Astrobiology and The Centre for Science at Extreme Conditions. With this in mind, further work should be carried out employing the same experimental design, however, with the use of microbial strains not isolated from a naturally high-pressure environment. Additionally, other multiple environmental factors should be considered, including alkali pH and sub-/supra-optimal temperatures that are common extremes in high pressure environments. Furthermore, microbial resistance to antibiotics share similar pathways to environmental parameters such as NaCl (%) or heavy metals, and subsurface communities are often areas of interest for antimicrobial resistance research (Dickinson et al., 2019; Harrison et al., 2017), understanding the net effect of these natural parameters could provide crucial information in antibiotics resistance studies.

6.5.3 Conclusions

The knowledge that there are more instances of life occupying habitats subject to higher than atmospheric pressures raises questions about the potential synergistic or antagonistic nature of combined environmental extremes in high-pressure environments. There is a body of scientific literature concerning the effects of high pressure on tolerance to other extremes such at pH and temperature individually, yet there is a lack of data exploring the interplay between multiple stresses under the physical constraints of high pressure compared with those same stresses under atmospheric pressure conditions. The resulting data of this study shows significant synergistic interactions between high pressure, NaCl and pH culture conditions where high pressure increases the model organisms capacity to deal with changes in environmental NaCl concentrations and pH, extending the boundaries of propagation for the model strain. These findings further highlight the need for a better understanding of life under concomitant extremes to provide a clearer definition of boundaries of habitability on Earth.

Chapter 7

Conclusion

The incentive for the research in this thesis was to contribute to the further understanding of the limits of life under multiple extremes. To address this, microbes were cultured under combinations of extreme environmental parameters that are often used to define the limits of life when experienced individually, which contrasts significantly with a more accurate characterisation of natural environments as defined by multiple physicochemical parameters. This chapter will summarise the principal findings of the individual studies of the thesis. Conclusions of experimental results and detailed comments on future work can be found at the end of each data chapter respectively.

7.1 Summary of main conclusions

The principal results of the work conducted in this thesis reveal the limits of life in extreme environments cannot be defined by single extremes imposed individually but when determining the habitability of a given environment, one must consider the net effect of multiple extremes. The boundary space within which biological processes occur is shaped by the interactions of multiple extreme conditions. Additionally, the physical parameters of high pressure and the presence/absence of oxygen further act to alter the boundaries of the habitability space on Earth. The experimental work in this thesis that demonstrate this core conclusion, as provided by each data chapter, are outlined here:

7.1.1 Chapter 4: Multiple extremes define narrower limits to microbial growth than individual extremes

The effects of extremes in NaCl concentration, pH and temperature experienced in isolation are well understood, yet despite the existing laboratory and field data and our understanding that natural environments can be best characterised by the net effect of multiple environmental parameters, basic studies on the interplay between concomitant environmental extremes on microorganisms is surprisingly limited. By assessing the effect of multiple extreme parameters on microbial propagation, Chapter 4 demonstrates that a combination of stresses of salt (NaCl), pH and temperature significantly limit life more than these parameters do when experienced individually. Further to this, the complex interactions of these environmental extremes act to limit microbial growth in different combinations. For example, pH dominated the final biomass over salt concentration at any given temperature where it can be seen that within the optimal salinity range of the model organism pH is the dominant determining factor, however, sensitivity to saline conditions was also amplified with increasing temperature where it is observed that supra-optimal temperatures increase sensitivity at all pH values tested. One explanation for these results could be through changes in cell membrane. Temperature, pH and salt adaptations involve structural changes in cellular membranes that act to increase or decrease ion-permeability and under multiple extremes these adaptations are shown here to interact antagonistically thereby reducing the limits of life under otherwise viable conditions. The findings provide insight in the multiplicative effect of common environmental stresses and highlights a need to better define the habitability space on Earth by taking into account the combined effect of concomitant stresses within natural habitats.

7.1.2 Chapter 5:The presence of oxygen may have only released some organisms from low energy anaerobic conditions

For an organism to grow and reproduce in a given environment it must be able to meet the energetic requirements necessary to deal with environmental stresses imposed on it and support a functioning biochemistry. Though there is an abundance of research identifying the limits of growth under individual environmental stresses for both aerobic and anaerobic strains, and our

understanding that energy yields differ between aerobic and anaerobic respiration, few laboratory studies have been done to systematically explore the interactions of three or more stresses on the limits of microbial growth under both aerobic and anaerobic conditions. With this in mind, three facultative anaerobic strains were used to assess the effect of multiple stresses of salt, temperature and pH on growth under both aerobic and anaerobic culture conditions. Chapter 5 demonstrates that when cultivated under oxic and anoxic conditions, the limits of microbial growth under multiple extremes were significantly different. Contrary to our understanding that aerobic respiration produces higher energy, the results from this chapter reveal the complex interactions of multiple environmental extremes have distinctly different effects when organisms are exposed to aerobic and anaerobic environments, where we see two of the model strains better able to deal with acidic conditions when cultured anaerobically compared with aerobic cultures. There is significant literature that covers the growth response of both aerobic and anaerobic organisms to extreme conditions, however, this research addresses a lack of comprehensive comparative data by using the same organism under combinations of environmental stresses to assess the differential growth response under the standard and theoretically energy limiting conditions. This has significant implications when considering the affect the rise of oxygen during The Great Oxidation Event may have had on Earths anaerobic microbial population. This newly exploitable energy source may have released some organisms from the constraints of low energy anaerobic conditions, but in certain environments, such as hydrothermal vent systems, life may have found little or no impact on extending the boundaries of life.

7.1.3 Chapter 6: the physical environmental parameter of hydrostatic pressure governs the limits of life under multiple extremes.

The effects of pressure are ubiquitous on Earth. Indeed, the majority of life occupies habitats that are subject to higher than atmospheric pressures, with the deep-sea piezosphere and subsurface environments predicted to be major habitats for prokaryotic life that far exceed numbers found elsewhere. High pressure environments are subject to multiple additional extremes, yet our understanding of the interplay between multiple stresses under the physical constraint on pressure is limited. The findings from chapter 6 show that

under a combination of stresses of salinity and pH, the physical parameter of hydrostatic pressure significantly changes the growth limits of the strain H. hydrothermalis. Under high pressure, this strain was better able to grow under acidic pH conditions that were shown to drastically reduce growth in experiments conducted in chapter 4. Additionally, when compared with growth at atmospheric pressure, *H. hydrothermalis* displayed higher growth under all combinations of salinity and pH used in this study. Further to the results reported in chapter 4, alteration in membrane structure may be one explanation for these data as high pressure and pH are known to be common physicochemical parameters that affect membrane integrity, increasing lipid packing and loss of permeability. Here we see pressure acting as a primary controlling factor for growth under pH and saline conditions. Though previous work has been conducted using the same model organism under similar conditions (pressure, temperature and salinity) (Kaye and Baross, 2004), the work in this study examines the effects of multiplicative extremes at increments that allow for a more robust picture of the changes in sensitivity to multiple extreme conditions. These data have significant implications in determining the habitability of a given environment, particularly when considering what constitutes an extreme condition to the organism in question.

7.1.4 Final remarks

The experiments presented in this thesis show multiple extremes restrict the limits of life more than single extremes imposed individually. The boundaries of the habitability space are determined by the multiple interactions between extreme conditions that prove both synergistic and antagonistic in certain combinations. For example, environmental pH can determine microbial growth parameters over salinity, but high temperature extremes act to govern microbial limits under a combination of these parameters. Synergistic interactions between the physical environmental extreme of high-pressure are revealed to extend the boundaries of habitability under multiple extremes conditions that are otherwise collectively antagonistic, further pushing the limits of life under concomitant extreme conditions. Further to this, energetic demands to deal with multiple extremes in the environment can be considered a physical environmental parameter that acts to determine the edges of the habitability space by reducing or expanding the limits of life for certain organisms and reducing the limits for others. Understanding how the multitude of environmental physico-chemical

extremes interact to determine the boundaries of habitability on Earth is crucial in furthering our understanding of the true limits of life. This knowledge would contribute significantly to areas of research such as origin of life studies, biotechnology, medicine, climate change and the search for life in the universe.

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